AN ABSTRACT OF THE THESIS OF

Brenda L. Wiens for the degree of Doctor of Philosophy in the Department of Zoology presented on October 21, 1992. Title: Neural Regulation of the Heart and Egg-Laying Behavior in the Nudibranch Mollusc *Archidoris montereyensis*.

Redacted for Privacy

Abstract approved: _____

Philip H. Brownell

In this thesis I describe neurons and neurotransmitter systems regulating the heart and egg-laying behavior of the nudibranch mollusc *Archidoris montereyensis*. Like many gastropods, neurons in this animal are comparatively few, and the larger of these can be identified individually and associated with discrete behavioral functions. The simplicity of these neuronal systems makes this an advantageous preparation for studying the diversity and functional significance of transmitter actions, especially in the context of complex behaviors.

The heart of *Archidoris* is predominantly controlled by a few motor neurons that have uniquely potent actions compared to those of other gastropods. The data presented in this thesis indicate that regulation of the heart involves many neurotransmitters, despite the simplicity of the cardiac circuit. I used an isolated heart bioassay to show that Serotonin, dopamine, and several neuropeptides (R15α2, small cardioactive peptide

B, myomodulin, and FMRFa) had excitatory actions, acetylcholine had inhibitory actions, while glycine, R15α1, and substance P had no observable effect on the heart. Most of these neurotransmitters appear to be released from discrete nerve terminals in the myocardium since neurons in the central nervous system and cardiac nerves exhibit immunoreactivity to these transmitters. The excitatory transmitters have similar, predominantly inotropic, actions on the isolated heart which raises new questions concerning their individual functions in cardiac regulation. Data presented here suggest that specific actions result from different thresholds of activity, anatomical differences in spacial patterns of release, and modulatory interactions between these neurotransmitters.

Egg-laying provides a behavioral context in which to investigate the physiological significance of these mechanisms. I show that egg-laying in *Archidoris* is triggered by a discrete set of neuroendocrine cells. These neurons, the Intercerebral White Cells, share morphological, biochemical, and electrophysiological characteristics with ovulogenic neurons in the gastropods *Aplysia californica* (bag cells) and *Lymnaea stagnalis* (caudodorsal cells). This suggests that the neuroendocrine systems controlling egg-laying are conserved; thus, as in these other gastropods, the neural correlates of this behavior may be evocable in experimental preparations of *Archidoris*. In summary, using *Archidoris*, we have the opportunity to examine the physiological mechanisms that coordinate heart activity during execution of a complex behavior.

Neural Regulation of the Heart and Egg-Laying Behavior in the Nudibranch Mollusc *Archidoris montereyensis*

by

Brenda L. Wiens

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Completed October 21, 1992

Commencement June 1993

Approved:

Redacted for Privacy

Associate Professor of Zoology in charge of major

Redacted for Privacy

Chair of Department of Zoology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented: October 21, 1992

Typed by Brenda Lynn Wiens

ACKNOWLEDGEMENTS

Completing this thesis would not have been possible without the help of many people. I would like to thank my advisor, Philip Brownell, for his support throughout my graduate career and particularly for encouraging me to pursue this doctoral degree. I also thank my committee members, Bob Becker, Fred Stormshak, Elaine Ingham, Frank Moore, and Tom Murray for their time and efforts. I especially appreciated their patience and flexibility concerning scheduling of exams.

I want to acknowledge and thank my colleagues in other labs who helped with this research. Naweed Syed, at the U. of Calgary Medical School, performed the chromatographic detection of biogenic amines, and John Bishop, Barry Masinovsky, George Mpitsos, Thomas O'Donohue, Seppo Soinila, and Klaudiuz Weiss generously gave me antisera which were essential for the immunocytochemical studies. Additionally, I am grateful to John Morris and those in his lab for sharing their photographic and histology equipment.

Other individuals have also helped me with aspects of this research. Several students, Ethan Kennedy, Michelle Schroeder, Anna Bandick, and Sylvia Owens assisted me with data analysis, poster preparations, and experiments. I especially appreciated Slyvia's work printing most of the pictures in this thesis. Further, many people risked life and limb in the intertidal to collect *Archidoris*. To all of you, I say a resounding thank

you. In this regard, I owe a special thanks to my husband, Greg, for going out every month, no matter what the time or weather. He is an *Archidoris*-spotter and bucket-carrier extraodinaire!

I couldn't begin to thank all of the faculty, staff, and graduate students in the Zoology Department who have provided encouragement and friendship. I would like to extend a special thanks to the second floor gang and to the past and present students in Phil's lab, Dylan Bulseco, Doug Gaffin, Scott Ligman, Jim Morgan, and Eric Webb. Thanks for all of your support and for making each and every day an interesting one.

Finally, I want to thank my husband, Greg, our families, and Sally and Todd Huffman for their ever present support, encouragement, and love. I know I couldn't have keep going without you.

I gratefully acknowledge the financial support of the National Institute of Mental Health, Sigma Xi, and the Zoology Department.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Neural Regulation of the Heart and Egg-Laying Behavior in the Nudibranch Mollusc *Archidoris montereyensis*

CHAPTER 1

INTRODUCTION

The ultimate goal of behavioral neurobiology is to understand how the nervous system regulates animal behavior. Studies of the neuronal basis of behavior are founded on the assumption that the output of the nervous system can be defined in terms of the properties of individual nerve cells and the functional interactions between these neurons. One area of intense research focuses on the functions of neurotransmitters as the chemical mediators of neuronal actions. Our appreciation of the potential complexity of neuronal signalling has grown over the past 15 years due to the identification of many novel neurotransmitters, particularly neuropeptides; the discovery that more than one transmitter can co-exist in the same neuron; and the characterization of multiple pre- and post-synaptic receptor subtypes and intracellular signalling mechanisms (Hokfelt, 1987; Bloom, 1988). These discoveries raise fundamental questions concerning the functions of individual transmitters and how diverse assemblages of transmitters released onto single targets produce

coordinated responses. In this thesis, I describe neurochemical regulation of the heart in the mollusc *Archidoris montereyensis* and propose that this preparation is an advantageous one for studying the function of multiple transmitters in a behavioral context.

It is evident from extensive studies of vertebrate animals that neurochemical regulation of the cardiovascular system offers a rich opportunity to investigate the physiological and behavioral significance of multiple transmitter interactions. First, cardiac output and vascular resistance are precisely controlled to maintain adequate circulation to active tissues (Ganong, 1989). Secondly, the behavioral output of the heart, its beat frequency and pressure pulse amplitude, are relatively easy to measure. Vascular blood flow is more difficult to assess but serves as a highly dynamic and precise measure of behavioral change. Thirdly, more than 20 neurotransmitters appear to mediate autonomic regulation of the mammalian cardiovascular system, including the classically described sympathetic and parasympathetic transmitters, norepinephrine and acetylcholine, as well as serotonin (Owman et al., 1987), purines (Burnstock, 1987; Pappano and Mubagwa, 1992), and many neuropeptides (Said, 1987). In this way the cardiovascular system seems to be a relatively simple motor system with quantifiable behaviors under the influence of a large number of neurotransmitters.

It seems reasonable to conjecture that no transmitter acts in

isolation on the heart of vascular tissues given the numbers of transmitters acting on these organs and the observation that many peripheral neurons innervating the heart and vasculature contain more than one transmitter (Dalsgaard et al., 1986; Said, 1987, Sundler et al., 1987). Transmitter interactions have been demonstrated on cardiovascular organs, although the physiological significance of even the most thoroughly described of these is not clear. For example, the first demonstration of a transmitter interaction was between the parasympathetic and sympathetic divisions of the autonomic nervous system in the heart. Studies using adrenergic or cholinergic antagonists during stimulation of cardiac nerves have shown that reciprocal antagonism occurs through both pre- and postsynaptic mechanisms. Presynaptically, acetylcholine released upon vagal stimulation blocks norepinephrine release by binding to muscarinic receptors on sympathetic nerve endings (Vanhoutte and Levy, 1980). Norepinephrine released from sympathetic nerves does not appear to inhibit the release of ACh, but neuropeptide Y, a peptide co-released with norepinephrine, does mediate presynaptic inhibition of ACh release (Potter, 1985; Warner and Levy, 1988; Manabe et al., 1991). Postsynaptic mechanisms for antagonism also exist through the opposing actions of muscarinic and ß adrenergic receptors on adenylate cyclase and ion channels mediated by the GTP-binding proteins Go and Gs, respectively (Yatani et al., 1990). Despite

our knowledge of these cellular and molecular mechanisms of action the physiological relevance of this interaction has yet to be demonstrated (Vatner, 1992). Function is difficult to assess because the neuronal sources of these transmitters in the central nervous system are not easily accessible except in highly reduced preparations. Under these conditions the endogenous patterns of neuronal activity during different behaviors cannot be mimicked or manipulated.

An alternative approach is to investigate transmitter interactions in the context of invertebrate cardiovascular regulation. In invertebrate preparations the activity of identified neurons can be manipulated and the responses of central and peripheral targets monitored in intact and functioning animals. Molluscan species are especially valuable models for study because their hearts are myogenic, like those of vertebrates, and this activity is regulated by relatively simple circuits of neurons in the central nervous system. Previous studies of neuronal regulation of cardiovascular functions have been conducted mostly in gastropod molluscs. Gastropods offer the best molluscan model for study because neurons in the central nervous system are easily identifiable and there is now a considerable body of information concerning the synthesis, release, and mode of action of neurotransmitters and hormones in these animals (Walker, 1986; Josse, 1988). In this introduction I will review what is known about neural control of the heart in gastropod molluscs and why

these preparations comprise an advantageous model for investigating the behavioral significance of transmitter actions and interactions.

NEURAL REGULATION OF GASTROPOD HEARTS

The myogenic rhythm of the gastropod heart, like that of the vertebrate heart, is regulated by both intrinsic and extrinsic factors. Myogenic contractions are thought to be initiated in the atrio-ventricular valve that divides the two chambers (atrium/ventricle) of the heart (Kuwasawa, 1979). Similar to the pacemaker cells in the mammalian sinoatrial node, the modified muscle cells of the atrio-ventricular valve contain less differentiated sarcomeres and are highly innervated (Jones, 1983; Kuwasawa, 1979). Little is known about intrinsic regulation of the heart, though Smith (1985) has shown that the heart of the prosobranch *Busycon canaliculatum* is very responsive to changes in diastolic preload pressure. Stroke volume and heart rate increase when the heart is stretched due to greater ventricular filling, similar to the Frank-Starling reflex in vertebrate hearts (Guyton, 1981). The importance of this control mechanism in an intact animal is unclear as large changes in returning venous pressure in an open circulatory system are not expected (Smith, 1990). In the opisthobranch mollusc *Aplysia californica*, though, a reduction in venous return stimulates the gill to contract (respiratory pumping), thus increasing venous return and cardiac output (Furgal and

Brownell, 1987). Extrinsically, the central nervous system modulates the rate and force of cardiac contractions through direct neural innervation and circulating neurohormones. Excitatory and inhibitory cardiac motor neurons have been identified in members of each molluscan subclass (prosobranchs, S.-Rosza, 1979a; pulmonates, S.-Rosza, 1979b, Furukawa and Kobayashi, 1987a,b, Buckett et al., 1990b,c; opisthobranchs, Mayeri et al., 1974, S.-Rosza et al., 1980, Arshavsky et al., 1990). These neurons and their functions have been best characterized in the opisthobranch *Aplysia californica*.

The Cardiac Circuit of *Aplysia californica*:

The cardiovascular system in *Aplysia* is regulated by a circuit of neurons located in the abdominal ganglion (Fig. 1.1). The heart is innervated by at least two general classes of neurons - motor neurons that have direct synaptic actions on cardiac myocytes and neurosecretory neurons that have paracrine or hormonal actions.

Five motor neurons, two inhibitors and three excitors, have distinct actions which are mediated by both conventional and peptide transmitters. The two heart inhibitor motor neurons, $LD_{HI1\ and\ 2}$, have similar transient inhibitory actions that are mediated by acetylcholine (Liebeswar et al., 1975). The heart excitor, RB_{HE}, stimulates increases in heart rate which are putatively correlated with increases in the force of

contraction. This neuron may have a tonic effect on heart rate as it discharges at a frequency of about 1 Hz in a dissected preparation of the animal. Mayeri and co-workers (1974) have further demonstrated that selective and reversible removal of this cell from the heart circuit by injection of hyperpolarizing current causes an 18% decrease in heart rate. However, other investigators recorded no significant change in heart rate after cutting the pericardial nerve (Dieringer et al., 1978) or completely removing the abdominal ganglion (Feinstein et al., 1977). The two additional heart excitors, LD_{HE} and L7, appear to affect the heart only transiently (Mayeri et al., 1974; Alevizos et al., 1989). These neurons are silent in experimental preparations and briefly affect rate and amplitude of cardiac contractions only when stimulated. L7 may play a unique role in integrating cardio-respiratory responses as it innervates the gill, siphon, and vasculature as well as the heart (Alevizos et al., 1989). Although the heart excitors, RB_{HE} and LD_{HE}, are both serotonergic (Liebeswar et al., 1975), their differential actions may be explained by the presence of co-transmitters. Skelton and co-workers (1990) have recently shown that RB_{HE} contains the peptide transmitter R15α2. One function of this peptide may be to modulate the heart's response to serotonin as the excitatory effects of RB_{HE} are blocked by an antagonist to serotonin (Liebeswar et al., 1975).

The activity in these excitatory and inhibitory cardiac motor

neurons is largely determined by two antagonistic interneurons that coordinate the activity of motor neurons innervating the heart and respiratory organs. Interneuron I (L10; Koester et al., 1974), inhibits the gill and excites the heart by simultaneously exciting RB_{HE} and inhibiting the LD_{HI} cells, and interneuron II (L25/R25 network; Koester et al., 1974; Byrne and Koester, 1978; Byrne, 1983; Koester, 1989), causes strong contractions of the gill and siphon and inhibits the heart by exciting the LD_{HI} cells and inhibiting RB_{HE}. Thus, the activities of these cells results in stereotyped motor responses involving changes in the activity of the heart. Interneuron I activity causes an increase in heart rate and a decrease in vasomotor tone which raises cardiac output; in contrast, interneuron II stimulates respiratory pumping by exciting motor neurons that contract the siphon and gill while inhibiting excitatory heart motor neurons. Behaviorally, gill and siphon contractions increase circulation of seawater through the mantle cavity and hemolymph through the relaxed heart and vasculature. Thus, circulatory and respiratory functions are regulated in a coordinated manner by a discrete set of motor and interneurons with fixed patterns of synaptic interaction which do not appear to require much transmitter diversity.

Nevertheless, the heart is also innervated by peptidergic neurosecretory cells. Processes of two types of peptidergic neurons have been identified in the heart. One type comprise neurons R7 and R8 which

are members of the R3-14 cluster of "white" cells in the abdominal ganglion (Rittenhouse and Price, 1986). All the neurons in this cluster are believed to utilize co-transmitters as they contain high concentrations of the transmitter glycine (McAdoo et al., 1978) and express a gene that encodes three neuropeptides (Nambu et al., 1983). Although activity in these neurons has not been shown to affect the heart, the transmitters they contain can affect cardiac activity. For example, glycine increases heart rate and frequency at millimolar concentrations (Sawada et al., 1984), and one of the peptides expressed in these cells, histidine-rich basic peptide, causes a dose-dependent increase in the amplitude of heart contractions at a threshold concentration of 100 nM (Campanelli and Scheller, 1987). The high concentration of these transmitters required for activity suggests that they may have modulatory actions similar to glycine's actions in the anterior aorta (Sawada et al., 1984). Processes of another neurosecretory neuron, R15, also exist in the heart (Rittenhouse and Price, 1985). As with R7 and R8, activity in this neuron does not appear to directly affect the heart, but one of the peptides it expresses, R15α1, causes a dose-dependent tonic contraction of the heart at a low threshold (below 250 nM; Alevizos et al., 1991a). The physiological role of this action on the heart is not known, but the fact that R15 innervates multiple targets (Rittenhouse and Price, 1985) and is activated during egg-laying (Alevizos et al., 1991b,c,d), suggests that R15α1 may integrate

cardiac function with other components of this behavior. The apparent lack of direct action of these neurosecretory neurons raises the possibility that the primary role of these cells may be to modulate the heart's response to motor neuronal stimulation.

Three other neuropeptide transmitters also appear to be involved in cardiovascular regulation, although the identity of neurons releasing these peptides is unknown. Small cardioactive peptide B (SCP_B) increases the rate and force of heart contractions by the same mechanism as 5HT (increasing cAMP concentrations) but through separate receptors (Wernham and Lukowiak, 1983; Lloyd et al., 1985). Although SCP_B could not be detected in the hemolymph of *Aplysia,* its actions were originally thought to be hormonal because it was not biochemically detected in the heart (Lloyd et al., 1985). Recently, though, Skelton and Koester (1991) have immunocytochemically detected SCP_B in nerves which innervate the auricle, atrio-ventricular valve, and cristae aorta of the heart suggesting that the original biochemical assays were not sensitive enough. Another molluscan cardioactive peptide, FMRFa, has also been located by immunocytochemical techniques within neuronal processes in the auricle, atrio-ventricular and aortic valves, and other cardiovascular tissues (Harris and Ono, 1990), but it appears to have little if any effect on contractile activity when applied to the heart alone (Lloyd et al., 1985). Finally, processes immunoreactive for myomodulin C, one

member of a peptide family recently purified and sequenced from the accessory radula closer muscle of *Aplysia*, densely innervate the pericardium and vasculature (Miller et al., 1991a). Taken together, these data indicate that a diverse assemblage of peptides may directly or indirectly (i.e., via modulatory actions) mediate neural control of the heart in *Aplysia*.

In summary, at the cellular level, regulation of the heart in *Aplysia* is accomplished by mechanisms similar in nature to those operating in vertebrates. The neuronal pools innervating the hearts of these phylogenetically divergent groups include fast-acting excitatory and inhibitory motor neurons containing conventional and peptide transmitters as well as neurosecretory neurons that may have predominantly modulatory actions. The functional and behavioral significance of this dual mode of action is not clear, but future insights into these unresolved questions will likely require more information about the physiological consequences of transmitter action and the behavioral capacities of the heart and vasculature.

Cardiovascular Regulation in *Aplysia* in the Context of Complex Behavior:

In *Aplysia* the central nervous system mediates changes in cardiovascular functions during feeding (Koch et al., 1984), respiratory (Koester et al.,1974), excretory (Koester and Alevizos, 1989; Skelton and

Koester, 1991), and egg-laying behaviors (Ligman and Brownell, 1985; Koester and Koch, 1987; Brownell and Ligman, 1992). Investigating the neural mechanisms of these changes in the context of egg-laying behavior is an especially promising experimental approach since the neural correlates of this behavior can be studied in dissected preparations of the animal. Egg-laying behavior is triggered by peptides released from a group of neuroendocrine cells, the bag cells, and involves stereotyped motor (Arch and Smock, 1977) and visceromotor (Brownell, 1983; Brownell et al., 1992) activities that result in the production and deposition of egg-masses.

The discovery that the bag cells of *Aplysia* contain hormone-like factors that induce egg-laying when injected into resting animals (Kupfermann, 1967) opened the way for detailed morphological, physiological, and biochemical characterization of neurons that trigger this complex behavior. The bag cells occur in bilateral clusters, comprised of 400 small cells each, located at the anterior margin of the abdominal ganglion (Frazier et al., 1967). These normally silent cells fire in a prolonged burst (30-60 min) of action potentials, the bag cell afterdischarge, immediately prior to the initiation of egg-laying behavior (Kupfermann and Kandel, 1970; Pinsker and Dudek, 1977). During this afterdischarge, the electrically coupled bag cells fire in unison, releasing peptide transmitters from neurosecretory terminals in the pleural-

abdominal connectives and vascularized sheath of the abdominal ganglion (Stuart et al., 1980). The bag cell peptides, which are the products of a single gene, include egg-laying hormone (ELH), α-, ß-, γ, and δ-bag cell peptides (BCPs), and acidic peptide (Scheller et al., 1983). Although all of the functions of these peptides are not known, both ELH and αBCP affect many neurons in the central nervous system (Mayeri et al., 1979a,b; Rothman et al., 1983a; Brownell, 1983; Brownell et al., 1992), and ELH acts directly on the ovotestes to stimulate ovulation (Coggeshall, 1972; Dudek et al., 1980; Rothman et al., 1983b). This system has been a profitable model for studying neuropeptide actions because the physiological release of endogenous peptides from the bag cells can be mimicked *in vivo* or *in vitro* by electrical stimulation of the bag cells.

Cardiovascular neurons are among those affected by bag cell activity. Although, the behavioral outcome of these actions have not been measured directly, some actions are strongly indicated by the known behavioral functions of the target neurons involved. The following cardiovascular adaptations are hypothesized based on the central and peripheral actions of bag cell peptides:

1. A transient decrease in ganglionic circulation and a large increase in systemic circulation as a result of the action of ELH on neuron R15. As previously discussed R15 is a multimodal neurosecretory neuron that innervates the heart as well as other central and peripheral targets.

Branton and colleagues (1978) showed that the frequency of endogenous bursting activity in R15 observed in the isolated ganglion increased upon a bag cell activation and application of ELH. Recently, however, Alevizos and co-workers (1991b) demonstrated that R15 is normally silent in intact animals and thus ELH may initiate activity in this cell specifically during egg-laying. Among its actions R15 stimulates L7 (Alevizos et al., 1991c), another multimodal neuron, and the L25/R25 interneurons (Alevizos et al., 1991b). One effect of R15 stimulation of L7 is to contract the pleuroabdominal connectives (Umitsu et al., 1987; Alevizos et al, 1991c). Because the blood supply to the abdominal ganglion is directed rostrally past the bag cells and into vascular channels along the pleuroabdominal connective, Alevizos and colleagues (1991c) hypothesize that constriction of these connectives may slow the diffusion of bag-cell peptides from the ganglionic vascular spaces, thus, prolonging their central actions. Concomitantly, systemic circulation may also be dramatically increased due to stimulation of respiratory pumping by the L25/R25 neurons. Although ELH has a prolonged effect on R15 (>1 hr; Branton et al., 1978), R15's effects are expected to be transient, because these actions, mediated by the peptide R15α1, desensitize within several minutes (Alevizos et al., 1991b and c).

2. A delayed tonic increase in heart rate mediated by L10. Bag cell peptides cause a biphasic response in L10 - inhibition lasting 5-10 minutes

followed by prolonged excitation lasting 1-2 hours (Mayeri et al., 1979b). Long-term excitation of L10 leads to the facilitation of synaptic potentials in RB_{HE}, the excitatory heart motor neuron innervated by L10 (Koester et al., 1974). Thus, heart rate and presumably cardiac output would increase to meet the higher circulatory demands of egg-laying activity.

3. Redirection of hemolymph flow. Bag cell activation causes a long-term increase in the contractile activity of two of the three major arteries leaving the heart, the anterior aorta and the gastroesophageal artery. Although activity in the vasomotor neurons increases during a bag-cell afterdischarge, these tonic contractions of the vessels appear to be hormonally mediated as they still occur following vascular denervation (Ligman and Brownell, 1985). The hormaonally elicited pattern of activity in the vasculature may redirect the pattern of circulation such that flow to feeding and early digestive organs would decrease, while flow to the ovotestes, reproductive tract, and nutrient storage organs (hepatopancreas) would increase during egg-laying (Ligman and Brownell, 1985; Brownell and Ligman, 1992).

In summary, cardiovascular changes during egg-laying in *Aplysia* are mediated by both synaptic and neurohormonal mechanisms. Many transmitters are potentially involved and may act simultaneously on the heart. For example, R15 stimulation by ELH may cause the release of both inhibitory and excitatory cardioactive transmitters through its

excitatory effects on the L25/R25 cells and L7, as well as its direct innervation of the heart. Are all of these transmitters simultaneously released by these neurons, or are they differentially released depending on the level of neuronal activity? If they are simultaneously released, at what level do they interact to elicit a coordinated cardiac response? What is the result on contractile activity of the heart? Despite the accessibility of neurons innervating the heart and a behavioral paradigm in which to investigate their actions, these questions have not been answered in *Aplysia* due to the difficulty of maintaining consistent, long-term cardiac activity in dissected preparations of the animal.

NEURAL REGULATION of the HEART of *ARCHIDORIS MONTEREYENSIS*

Our previous studies (Wiens and Brownell, 1990a) of neural control of the heart in *Archidoris montereyensis* indicate that the cardiac neuromuscular preparation of this mollusc offers several advantages. First, the entire central nervous system of *Archidoris* is contained within one circumesophageal ganglionic ring with a thin, transparent sheath. It is possible, therefore, to access all of the essential neuronal elements of the cardiac circuit using standard intracellular recording techniques. Secondly, the cardiovascular organs are easily exposed by dissection, and the heart maintains consistent rhythmic beating for many hours without internal perfusion as required for the *Aplysia* heart preparation. Thirdly,

the cardiac motor neurons we have identified appear to be very strongly coupled to the heart and exhibit more consistent actions than those observed for the motor neurons in *Aplysia*. Lastly, the two most potent cardiac motor neurons in *Archidoris* can be found with a greater regularity than the cardiac motor neurons in *Aplysia*.

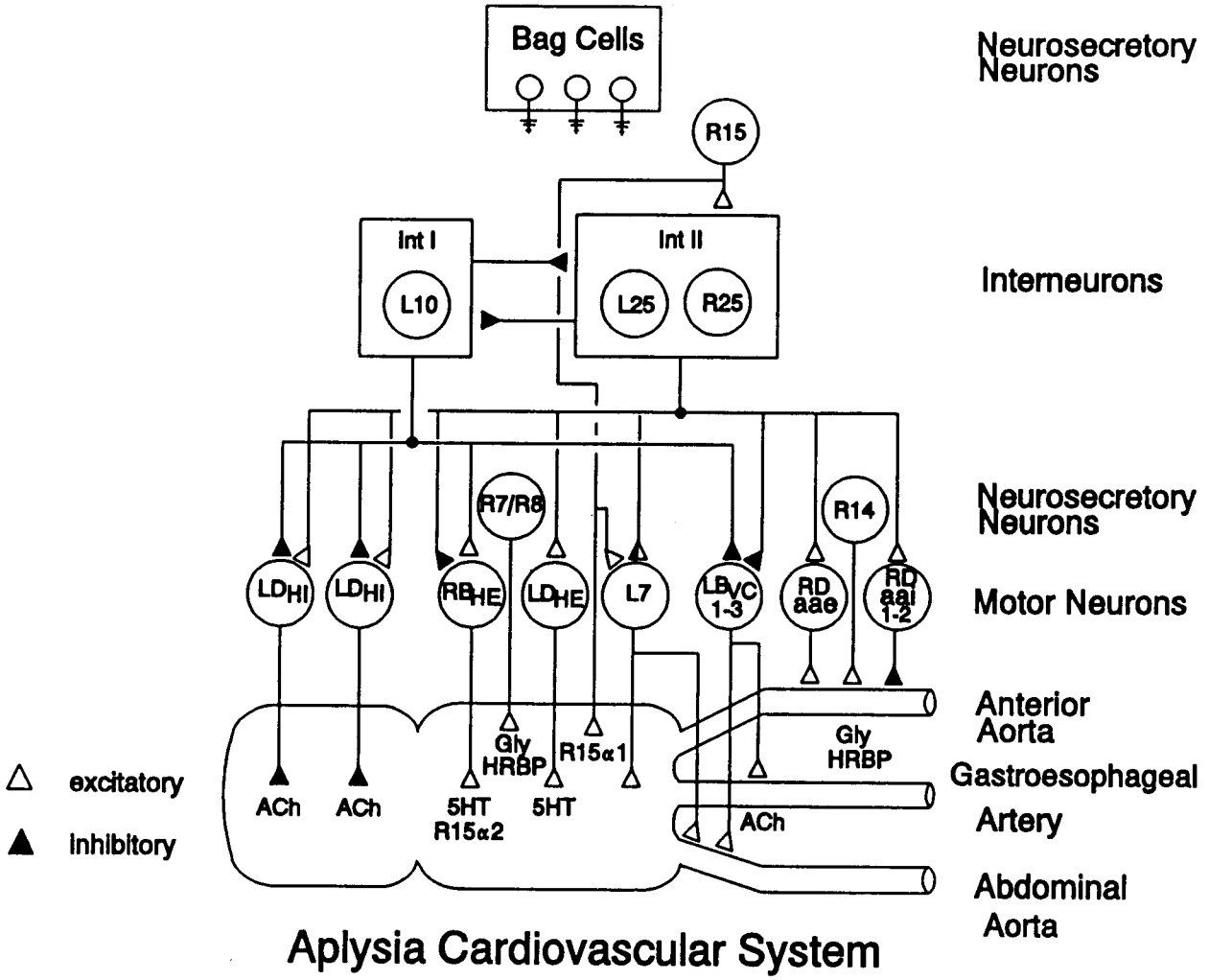
The motor component of the circuit of neurons innervating the heart of *Archidoris* consists of at least five motor neurons in the right pleural and visceral ganglia that innervate the heart via arterial and ventricular branches of the pericardial nerve (Fig. 1.2). The pleural ganglion motor neurons, one excitor and one inhibitor (PI_{HE} and PI_{HI}, respectively), have unusually powerful actions on the heart. Small fluctuations in the spontaneous activities of PI_{HE} and PI_{HI} noticeably affect contractile activity of the heart (Fig. 1.3). Spontaneous activity in PI_{HE} is correlated with an increase in the amplitude of ventricular contractions, and when stimulated to fire at greater frequencies, it also increases rate and tonus of contractions. The potency of PI_{HE} is most evident when only the auricle of the heart is beating with the ventricle near its threshold for contraction. In these circumstances a single action potential elicited in PI_{HE} can cause a sustained increase in ventricular contractions lasting many seconds (Fig. 1.3A, inset). These actions suggest that transmitters released from PI_{HE} have both short and long-term actions on the heart. Activity in PI_{HI}, the other potent heart effector, is closely correlated with bouts of cardiac

inhibition. These periods of inhibition can be reversibly eliminated by hyperpolarization of PI_{HI} indicating that this single neuron has a dominant role in regulating inhibition of the heart. Additionally, PI_{HI} has a tonic action on the heart which does not appear to accommodate during prolonged periods of PI_{HI} activity. In comparison to other molluscan cardiac motor neurons described in the literature, those in the pleural ganglion of *Archidoris* are the most potent heart-regulatory neurons identified. In dissected preparations of *Aplysia*, for example, the excitatory and inhibitory heart motor neurons must discharge at relatively high rates to produce significant effects on the heart (Mayeri et al., 1974). In terms of their strength of actions these neurons are more comparable to the cardiac motor neurons, V_{HE} and $V_{HI1\ and\ 2}$, in the visceral ganglion of *Archidoris* which are much weaker cardiac effectors than PI_{HE} and PI_{HI} (Fig. 1.4).

Thus, unlike most other invertebrate or vertebrate systems described to date, the cardiac motor neurons in *Archidoris* are especially effective in regulating the activity of the heart. Single neurons have immediate actions with short and long-term components. Furthermore, the close correlation between activity in the pleural motor neurons and activity of the heart indicates the unique importance of these neurons in cardiac regulation and suggests that neural pathways affecting circulatory function will impinge upon these few cells.

In this thesis I have expanded my investigation of this unusual cardio-regulatory system. In chapters two and three I identify putative cardioactive transmitters in *Archidoris* - chapter two shows that a variety of amine and neuropeptide transmitters have specific actions on isolated preparations of the *Archidoris* the heart, and chapter three describes the distribution of these transmitters in the central nervous system and peripheral nerves innervating the heart. Chapter four characterizes a group of neuroendocrine cells in the central nervous system, similar to the bag cells in *Aplysia*, that appear to trigger egg-laying behavior in *Archidoris*. Taken together these chapters provide the basis for investigations into the function of multiple transmitters in the context of cardiovascular regulation during a complex behavior.

Figure 1.1 Schematic diagram of cardiac innervation in *Aplysia*. The heart of *Aplysia* is innervated by fast-acting excitatory and inhibitory motor neurons, as well as neurosecretory neurons whose actions are unknown. Several levels of neural control exist. The activities of neurons directly innervating the heart are controlled by higher-order interneurons as well as neurosecretory cells that release their peptide transmitters non-synaptically.



Aplysia Cardiovascular System

Figure 1.1

Figure 1.2 Schematic diagram of cardiac innervation in *Archidoris*. The circumesophageal ganglia (C, cerebral; Pd, pedal; Pl, pleural; R, rhinophore; V, visceral) and heart are shown from a dorsal perspective. Five motor neurons regulate contractile activity of the heart - a heart excitor and an inhibitor in the right pleural ganglion and an excitor and two inhibitors in the visceral ganglion (inset). These neurons innervate the heart primarily via the arterial and ventricular branches of the pericardial nerve (PN). The walls of the heart are spread away from a dorsal longitudinal incision to reveal the general patterns of innervation along the ventral lumenal myocardium.

Figure 1.2

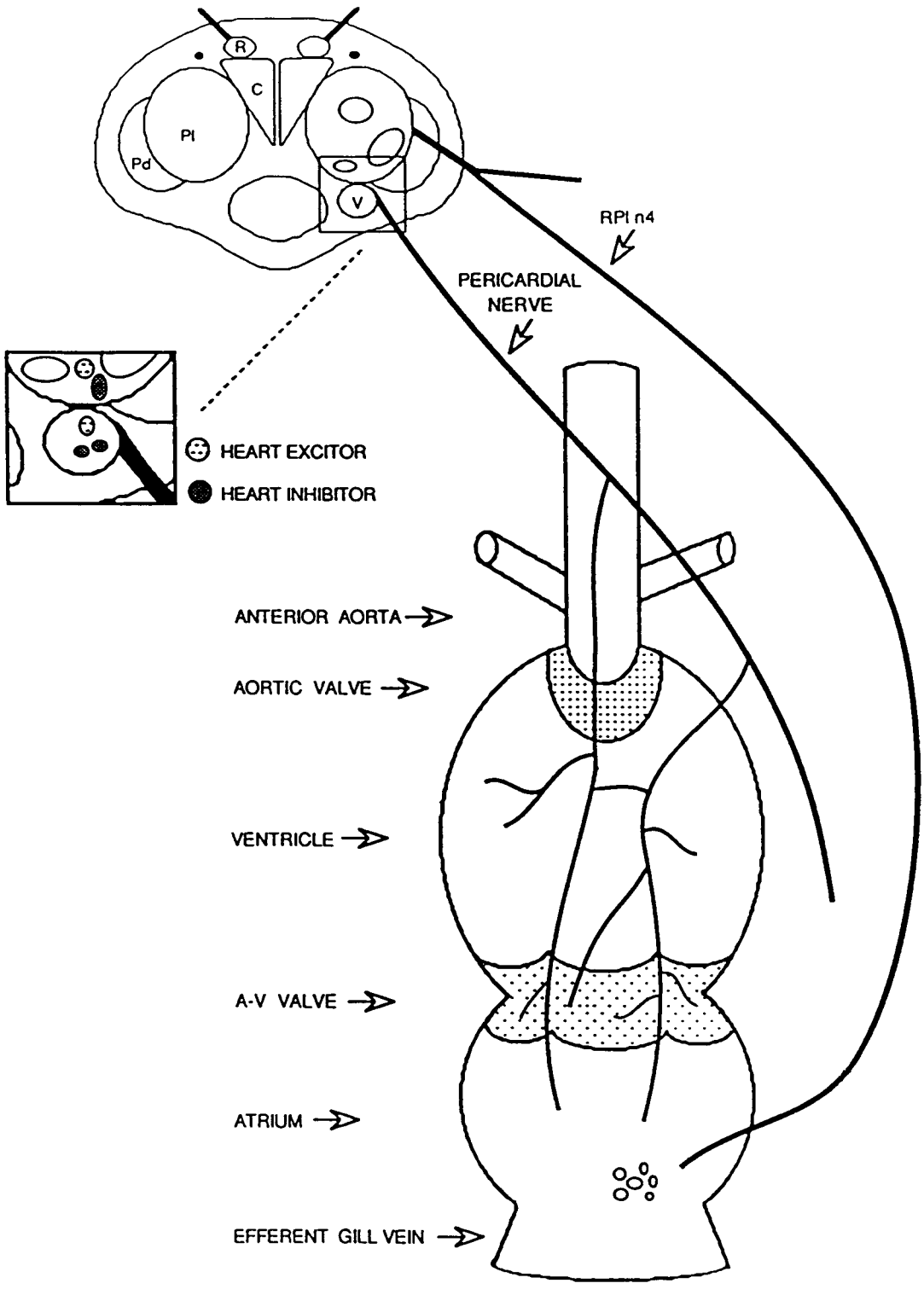

R
C
Pl
Pd
V
RPl n4
PERICARDIAL NERVE
HEART EXCITOR
HEART INHIBITOR
ANTERIOR AORTA
AORTIC VALVE
VENTRICLE
A-V VALVE
ATRIUM
EFFERENT GILL VEIN

Figure 1.3 Actions of pleural heart motor neurons in *Archidoris*. A. Cardio-excitatory actions of the pleural heart excitor motor neuron, Pl_{HE} - 1. Fluctuations in spontaneous activity of Pl_{HE} (lower trace, average spike rate 0.3-0.5 Hz) were positively correlated with changes in amplitude of ventricular contractions (upper trace) recorded by a tension transducer attached to the heart. When spontaneous activity of Pl_{HE} was reversibly eliminated by injection of hyperpolarizing current (horizontal line) the stronger ventricular contractions ceased. 2. Depolarization of Pl_{HE} (bars beneath trace) increased spike activity of the cell (average frequency indicated beneath bars) and stimulated contractile activity of the heart. Inset: Single or double action potentials (double arrow) stimulated in Pl_{HE} evoked long-term increases in strength of ventricular contraction. B. Cardio-inhibitory actions of neuron Pl_{HI} - 1. Spontaneous fluctuations in spike activity of the motor neuron Pl_{HI} were concurrent with decreased activity of the heart. Hyperpolarization of Pl_{HI} by direct current injection (horizontal line) raised the average frequency of heartbeat and eliminated recurrent periods of cardiac inhibition. 2. When Pl_{HI} was stimulated to fire at rates above 1 Hz activity of the heart was completely inhibited (average Pl_{HI} spike frequencies are indicated below each stimulus). Asynchronous contractions of the atrium and smaller amplitude ventricular contractions appear as a thickened baseline in these tension recordings. (Modified from Wiens and Brownell, 1990a)

Figure 1.3

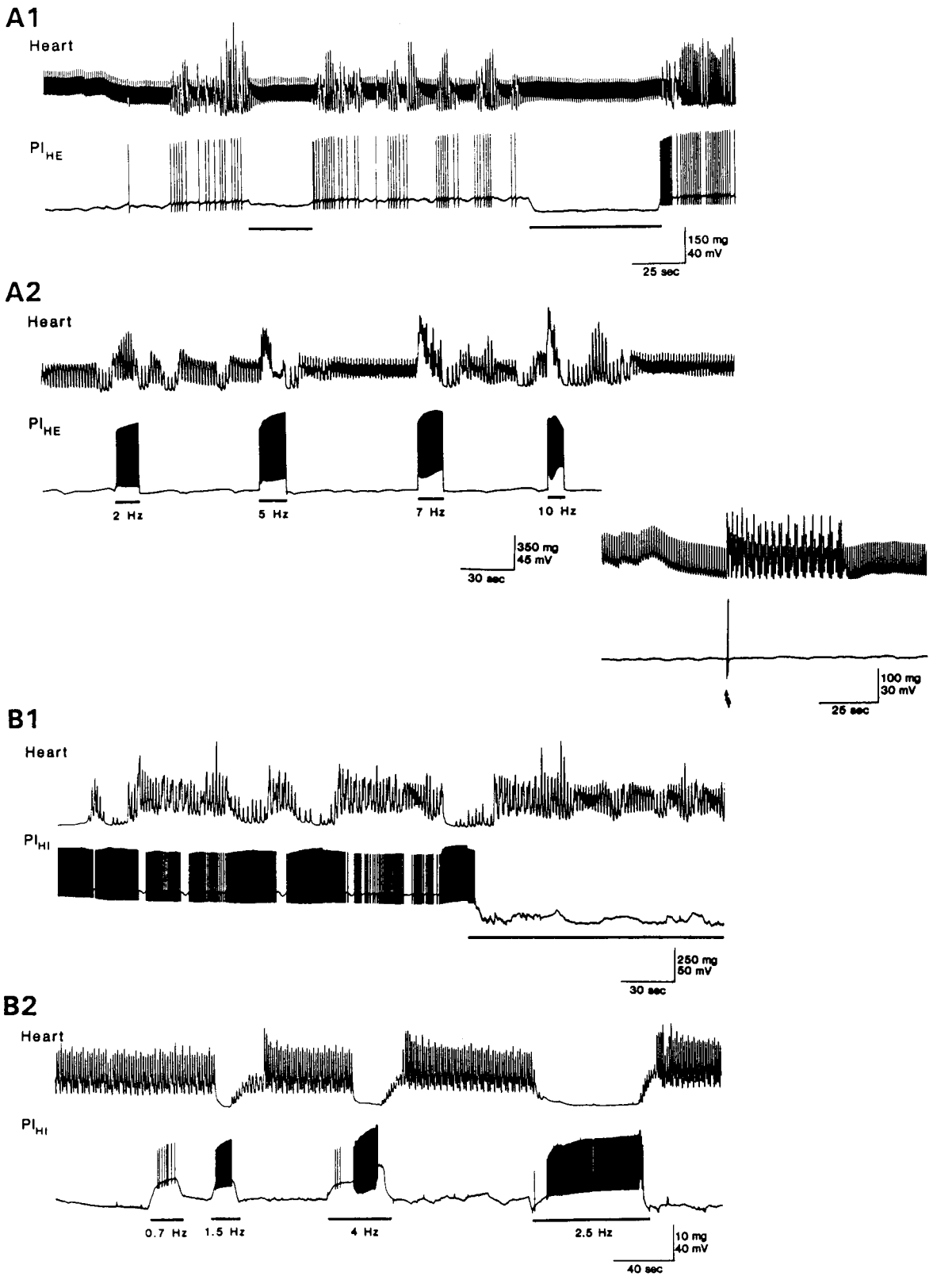
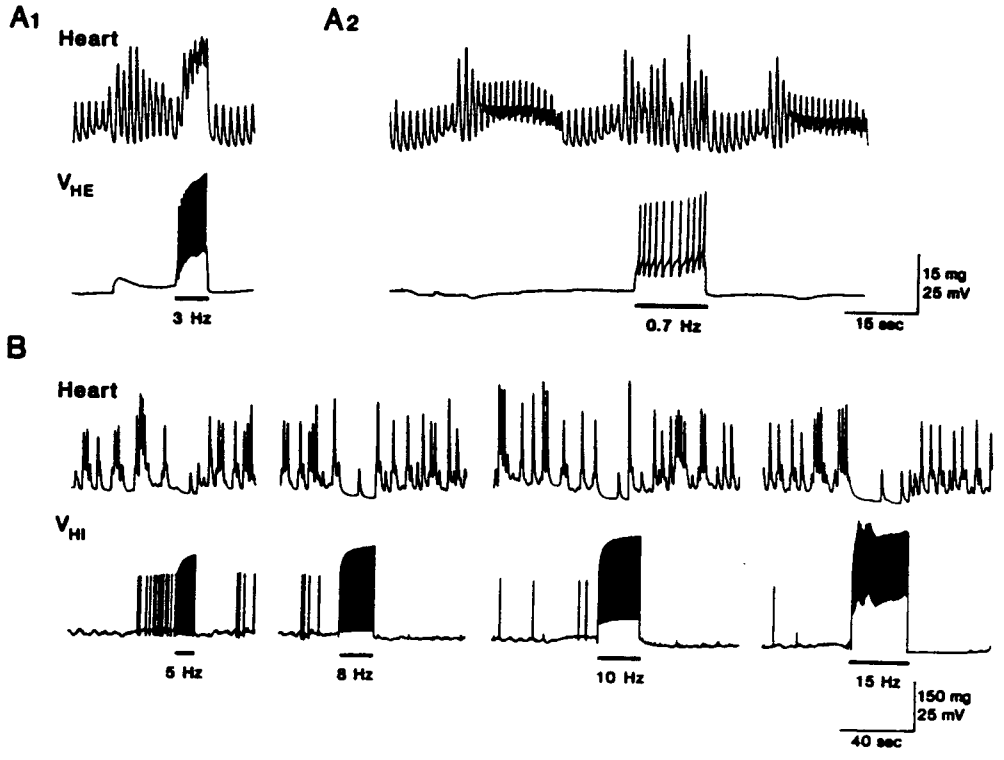

A1
Heart
PI HE
150 mg
40 mV
25 sec
A2
Heart
PI HE
2 Hz
5 Hz
7 Hz
10 Hz
350 mg
45 mV
30 sec
100 mg
30 mV
25 sec
B1
Heart
PI HI
250 mg
50 mV
30 sec
B2
Heart
PI HI
0.7 Hz
1.5 Hz
4 Hz
2.5 Hz
10 mg
40 mV
40 sec

Figure 1.4 Actions of visceral heart motor neurons in *Archidoris*. A. High rates of stimulated spike activity in V_{HE} increased the frequency and amplitude of cardiac contractions (A1); milder stimulation of V_{HE} prolonged spontaneous bouts of large amplitude ventricular contractions (A2). B. Stimulated spike activity in a V_{HI} cell reduces the frequency and amplitude of heart contractions. (Modified from Wiens and Brownell, 1990a)


A1
Heart
VHE
3 Hz
A2
0.7 Hz
15 sec
15 mg
25 mV
B
Heart
VHI
5 Hz
8 Hz
10 Hz
15 Hz
150 mg
25 mV
40 sec

CHAPTER 2

NEUROTRANSMITTER ACTIONS ON THE HEART OF THE NUDIBRANCH *ARCHIDORIS MONTEREYENSIS*

ABSTRACT

The heart of the mollusc *Archidoris montereyensis* is uniquely sensitive to regulation by cardiac motor neurons. The purpose of this study was to assess the sensitivity of isolated preparations of the heart to amines and neuropeptides known or suspected to mediate the actions of other gastropod cardiac motor neurons. In all we have examined cardiac responses to nine transmitters. Each transmitter was perfused through denervated, isolated preparations of the *Archidoris* heart as the rate, amplitude, and tonus of cardiac contractions were monitored. The amines serotonin (threshold < 10nM) and dopamine (100nM) and the peptides R15α2 (3nM), small cardioactive peptide B (10nM), myomodulin (0.8μM), and FMRFamide (20μM), were all excitatory. Although their thresholds of activity varied, all of these transmitters increased the rate and amplitude of heart contractions, and the amines and FMRFa increased diastolic tonus as well. The actions of these excitatory transmitters, with the exception of myomodulin, were similar in that the inotropic component of the response was more pronounced at all concentrations. Myomodulin also exhibited

modulatory actions as subthreshold concentrations potentiated the inotropic response of the heart to serotonin. Acetylcholine (10 nM) was the only inhibitory transmitter, decreasing the rate, amplitude, and tonus of contractions. The amino acid glycine and the peptides substance P and R15α1 had no affect on these parameters of contraction. Thus, despite the simplicity of the cardiac motor circuit, the heart of *Archidoris* appears to have receptors for a diversity of both conventional and peptide transmitters.

INTRODUCTION

Our perceptions of chemical mediation of neuronal activity have evolved over the last decade due to the identification of many novel neurotransmitters and the observation that more than one transmitter can mediate the actions of a single neuron. Invertebrate muscle preparations are profitable models for investigating the functional significance of multiple transmitter innervation of a single target because both the target tissue and the individual neurons innervating it are accessible for manipulation (for review see Calabrese, 1989). The heart of gastropod molluscs may be a particularly suitable preparation for several reasons. First, neuronally mediated changes in cardiac output are an essential component of several behaviors (Koester et al., 1974; Feinstein et al., 1977; Byrne and Koester, 1978; Dieringer et al., 1978; Koch et al., 1984; Grega and Prior, 1985;

Arshavsky et al., 1990). Secondly, estimates of cardiac output can be obtained from easily quantifiable parameters such as the rate and amplitude of contractions. Thirdly, the myogenic activity of gastropod hearts is regulated by the central nervous system via both excitatory and inhibitory motor neurons as well as neurosecretory neurons (Mayeri et al., 1974; S.-Rosza, 1979a,b; S.-Rosza et al, 1980; Rittenhouse and Price, 1985,1986; Campanelli and Scheller, 1987; Furukawa and Kobayashi, 1987a,b; Arshavsky et al., 1990; Buckett et al., 1990b,c; Alevizos et al., 1991a; Kerkhoven et al., 1991; Skelton and Koester, 1991). Lastly, several classes of neurotransmitters appear to mediate neuronal regulation of the heart, including aminergic, cholinergic, and peptide transmitters (Walker, 1986). In this and the following paper we characterize neurochemical regulation of the heart in the nudibranch gastropod *Archidoris montereyensis* and propose that this preparation is advantageous for studying the behavioral significance of multiple transmitter interactions.

Neurochemical regulation of gastropod hearts, like those of vertebrates, is complex. The amines serotonin (5HT) and acetylcholine (ACh) function as excitatory and inhibitory cardiac transmitters, respectively, in most molluscs (Mackay and Gelperin, 1972; Hill, 1974; Liebeswar et al., 1975; Kuwasawa, 1979; Boyd et al., 1985; Kobayashi, 1987; Smith and Hill, 1987; Kobayashi and Muneoka, 1989). Dopamine (DA) is also cardio-excitatory in pulmonates (Mackay and Gelperin, 1972;

Buckett et al., 1990a) and opisthobranchs (Wernham and Lukowiak, 1983), and the amino acid glycine excites the heart of the opisthobranch *Aplysia californica*, albeit at millimolar concentrations (Sawada et al., 1984). Many neuropeptides act on gastropod hearts, but the actions of most were assayed in a limited number of species. For example, histidine-rich basic peptide (Campanelli and Scheller, 1987), R15α1, and R15α2 (Alevizos et al., 1991a), excite the heart and myomodulin excites the pericardium of *Aplysia* (Miller et al., 1991); substance P excites the hearts of the pulmonates, *Lymnaea stagnalis* (Buckett et al., 1990a) and *Helix aspersa* (Boyd et al., 1985); and achatin-I excites the heart of the pulmonate *Achatina fulica* (Fujimoto et al., 1991). Two peptides, small cardioactive peptide B (SCP_B) and FMRFamide (FMRFa) have been assayed more broadly. While SCP_B has similar cardio-excitatory actions in pulmonates and opisthobranchs (Lloyd, 1982; Welsford and Prior, 1991), the heterogenous actions of FMRFa between gastropod subclasses (Lloyd et al., 1985; Kobayashi, 1987; Buckett et al., 1990a) suggest that the cardiac effects of peptides may not be as consistent between gastropod groups as those of aminergic transmitters.

As in other systems, the physiological relevance of having many transmitters converging onto one target tissue is unclear. Although some of these transmitters have been shown to mediate actions of motor neurons innervating the heart (Liebeswar et al., 1975; Buckett et al.,

1990b,c), their functions remain undefined because little is known regarding the behavioral states in which these neurons are active. For example, Skelton and Koester (1991) recently discovered that one of the heart excitatory motor neurons in *Aplysia*, RB_{HE}, synthesizes the peptide R15α2 as well as 5HT (Liebeswar et al., 1975). The function of this peptide is not known, but it does not appear to act alone because the excitatory effects of RB_{HE} are completely blocked by a serotonergic antagonist. Few studies have investigated possible simultaneous actions of transmitters on the heart. Synergistic and modulatory actions have been reported for the peptides AVT (Wernham and Lukowiak, 1983) and FMRFa (unpublished results reviewed in Kobayashi and Muneoka, 1989), respectively, on the heart of gastropods although the mechanism and physiological significance of these interactions is not known.

The heart of *Archidoris montereyensis* may be a useful preparation in which to address questions concerning the behavioral significance of transmitter actions. The advantage of this preparation is that the heart is regulated by motor neurons that have uniquely potent actions compared to other molluscan cardiac neurons. Previously, we identified 5 cardiac motor neurons in the central nervous system of *Archidoris* (Wiens and Brownell, 1990a). Two of these, an excitor and inhibitor in the pleural ganglion, appear to be the predominant effectors of cardiac activity, as low levels of spontaneous activity in either neuron significantly alters the amplitude and

rate of ventricular contractions. Our present goal is to identify the transmitters that mediate neuronal control of the heart in *Archidoris*.

In this paper we characterize the actions of molluscan cardioactive transmitters on an isolated preparation of the *Archidoris* heart as a first step toward identifying potential endogenous cardio-regulatory transmitters. The transmitters assayed were the aminergic transmitters, 5HT, DA, glycine, and ACh; and the peptides SCP_B, FMRFa, substance P, R15α1, R15α2, and myomodulin. This group of transmitters includes those that are cardioactive in all gastropods assayed as well as some which have only been assayed in members of a particular gastropod group. Preliminary reports of some of these findings have appeared (Wiens and Brownell, 1989, 1990b, 1991).

MATERIALS AND METHODS

Animals: *Archidoris montereyensis*, ranging from 3 to 8 cm in length, were collected year-round from rocky intertidal areas along the central Oregon coast. They were maintained in a re-circulating, natural seawater aquarium (13-15° C; 16L/8D photoperiod) and fed their native food, the sponge *Halicondria* sp. Only animals that responded normally to tactile stimulation were used for these experiments.

Isolated Heart Assay: The actions of transmitters on the *Archidoris* heart were determined using an isolated preparation of this organ. The heart and

anterior aorta were dissected from animals anesthetized by injection of isotonic $MgCl_2$ into their hemocoels (approximately 25% of body weight) and placed horizontally in a 5 ml plexiglass chamber lined with Sylgard (Dow Corning, Corp.). A peristaltic pump was used to internally and externally perfuse the heart at a constant rate with aerated, buffered (5 mM Hepes, pH 7.8) artificial seawater (ASW) enriched with 0.2% glucose, Eagles Minimum Medium Essential (0.2X) and Non-Essential (0.2X) amino acid and vitamin solutions (0.5X; Gibco). The experimental chamber and perfusion tubes were embedded in an aluminum block anchored on top of a thermoelectric device (Cambion 806-1036) to maintain the preparation and the perfusion medium at a constant temperature of 15°C. The heart was cannulated through the atrium and a three-way sample injection valve was used to introduce 80 μl of the assay sample into the internal perfusion stream without changes in flow or pressure. Prior to infusion, frozen stock solutions were serially diluted in ASW and assayed on the heart in ascending concentrations (DA solutions were made immediately prior to use to limit oxidation). ASW was perfused between each test application until the heart returned to a stable state. The transmitters assayed in this study were 5HT, DA, glycine, ACh, and substance P (Sigma Chemical Co.); SCP_B, FMRFa, and myomodulin A (Peninsula Laboratories, Inc.); R15α1 and R15α2 (gifts from K.R. Weiss, Mt. Sinai School of Medicine). Contractile activity of the heart was monitored with a tension transducer (Cambridge

Technology Model 400) attached to the ventricle by 7-0 suture thread and a stainless steel hook. The data were recorded on chart (Gould 2400s) and FM tape (Hewlett Packard 3968A) recorders and later analyzed for beat frequency, amplitude, and tonus (diastolic tension). Dose-response curves of transmitter concentration versus normalized rate and amplitude of peak response (minute with highest average response) were generated using a nonlinear least squares curve-fitting program (Graphpad Inplot, Graphpad Software, Inc.). Threshold was defined as the lowest concentration to cause detectable changes in any of the response parameters, and it was calculated for each transmitter as the average obtained for all applications. The concentration that caused the half maximal response (EC_{50}) was determined from the dose-response curve.

RESULTS

The isolated heart preparations used in this study maintained activity for approximately 8-10 hrs. Consistent contractile activity was maintained by applying a distending amount of tension, however, this often decreased preparation longevity as a result of tissue damage. The type of response (i.e., excitatory or inhibitory) to a particular active transmitter was consistent between animals, but the magnitude of the normalized responses to a particular concentration exhibited variability. The duration of these responses was approximately equivalent to the period that the

transmitter was present. Additionally, we did not observe any significant difference between the durations of conventional and peptide transmitter actions measured at equivalent concentrations (EC_{50}).

Actions of Conventional Transmitters

The responses of the isolated heart to glycine, 5HT, DA, and ACh are summarized in Table 2.1. Glycine had no effect at or below the highest concentration tested (1 mM), while the monoamines, 5HT and DA, elicited dose-dependent excitatory responses that included increases in the rate, amplitude, and diastolic tonus of heart contractions. The dose-response curves for these transmitters (Fig. 2.2, A and B) and the form of the heart's response to each transmitter (Fig. 2.1, A and B) were nearly identical, but the potency of 5HT was ten-fold greater (threshold <10nM vs. <100nM). These transmitters predominantly affected contraction amplitude, increasing it as much as three-fold while heart rates increased only modestly.

ACh was the only transmitter with an inhibitory effect on the heart. Infusion of this transmitter into the heart decreased the rate, amplitude, and tonus of the myocardium (Fig. 2.1, C), and completely inhibited contractions at concentrations above 1 μM. The rate and amplitude responses were dose-dependent over a similar concentration range (Fig. 2.2, C).

Actions of Peptide Transmitters

The peptides, R15α2, SCP_B, myomodulin, and FMRFamide, excited the heart (Figs. 2.3 and 2.5), while R15α1 and substance P, had no effect at or below concentrations of 10 μM and 100 μM, respectively (Table 2.1). R15α2, with an average threshold of <3 nM, was the most potent cardio-excitatory transmitter assayed. The rate and amplitude of contractions both increased over the same concentration range, but, like the monoamines, R15α2 predominantly increased amplitude of heart contractions, increasing this parameter as much as two-fold (Fig. 2.4, A). SCP_B, with a threshold of <10 nM, was less potent than R15α2, but its cardio-excitatory actions also mimicked those of the monoamines (Fig. 2.4, B).

Myomodulin and FMRFamide exhibited the least potent actions, having thresholds of <0.8 μM and <20 μM, respectively. Unlike the other excitatory transmitters, micromolar levels of myomodulin had a greater effect on the rate than on the amplitude of heart contractions at higher concentrations (Fig. 2.4, C). FMRFa induced dose-dependent increases in the rate and amplitude of heart contractions at relatively high ($> 20\mu$M) concentrations (Fig 2.5). Additionally, FMRFa was the only peptide that consistently affected the tonus of heart contractions.

Transmitter Interactions

In other gastropod muscle preparations, often transmitters that exhibit high thresholds of action or have no activity when assayed alone modulate the muscle's response to other transmitters (Weiss et al., 1978; Hall and Lloyd, 1990). We assayed for possible modulatory actions of FMRFa and myomodulin, two peptides with high thresholds of action, by conducting two types of experiments. In the first set of experiments we assayed the ability of FMRFa or myomodulin to modulate stimulated contractions of the heart. Cardiac contractions were evoked in quiescent heart preparations by suction electrode stimulation of the pericardial nerve while subthreshold concentrations of transmitters were perfused through the heart. None of the contraction parameters measured - rate, amplitude, tonus, or form - were affected by transmitter application (data not shown). A caveat of these experiments, though, is that nerve stimulation may cause the release of endogenous peptides, potentially masking the effects of exogenously applied peptides.

In the second type of experiment we assayed the ability of FMRFa or myomodulin to modulate the effect of 5HT on spontaneous cardiac contractions. The peptides were assessed by comparing the heart's response to a submaximal concentration of 5HT applied with and without subthreshold levels of FMRFa or myomodulin. Normally successive applications of the same concentration of 5HT applied to the heart caused

the magnitude of the response to decrease (Fig. 2.6, A). Therefore, the heart was first exposed to multiple applications of 5HT, to confirm this desensitizing trend, before the putative modulatory transmitter was applied. We observed no change in any of the response parameters when sub-threshold concentrations of FMRFa were applied with 5HT (Fig. 2.6, B). In contrast subthreshold concentrations of myomodulin potentiated the inotropic response of the heart to 5HT (Fig. 2.6, C). Simultaneous application of 10 nM myomodulin with 10 nM 5HT temporarily reversed the response desensitization to 5HT and returned the normalized peak inotropic response of the heart to initial levels (160% control). Two additional transmitters, SCP_B and glycine, did not affect the cardiac response to 5HT (data not shown).

DISCUSSION

The large number of transmitters active on the heart of *Archidoris*, indicates that neurochemical regulation of this heart, like that of other molluscs, approaches the complexity observed in vertebrate cardiac regulation (Said, 1987). As in other gastropods the biogenic amines, 5HT and DA, and ACh have excitatory and inhibitory actions, respectively. The group of peptides active on the heart of *Archidoris* is unique to this species and includes R15α2, SCP_B, FMRFa, and myomodulin, which are all excitatory.

Aside from differences in potency, the actions of all the excitatory transmitters, except myomodulin, are similar in that they maximally affect the amplitude of contractions. The parallel actions of the biogenic amines and the peptide SCP_B are qualitatively similar to those observed on other gastropod muscles (Abrams et al., 1984; Lloyd et al., 1984, 1985). The similar actions of these transmitters suggest that they may have common mechanisms of action on the *Archidoris* heart as they do on other gastropod muscles. The actions of these transmitters on the heart of *Aplysia*, for example, are mediated through activation of the same second messenger system (cAMP) by two classes of receptors, one specific for the amines and the other for SCP_B (Wernham and Lukowiak, 1983; Drummond et al., 1985, Lloyd et al., 1985).

The physiological consequences of these actions are unknown and may be different for isolated versus intact hearts. The predominantly inotropic effects of most of the excitatory transmitters and the capacity of the heart to exhibit large changes in contraction amplitude suggest that this parameter may be central to altering cardiac output in *Archidoris*. This conclusion is consistent with results obtained in other molluscs which show that an increase in stroke volume and not heart rate is the major factor increasing cardiac output (Smith, 1990). We did not directly measure cardiac output in *Archidoris*, but concentrations of transmitters

that caused large increases in the strength of contraction without a comparable increase in rate would be expected to increase output.

The individual actions of most of these transmitters are not identical to those of the heart motor neurons in *Archidoris*. Although ACh mimics the actions of the pleural heart inhibitor, Pl_{HI}, none of the excitatory transmitters completely mimics the actions of the pleural heart excitor, Pl_{HE}. When spontaneously active, Pl_{HE} increases the amplitude of heart contractions. This effect is reminiscent of the predominantly inotropic actions of most of the excitatory transmitters, however, these transmitters also have chronotropic actions that are not elicited by low levels of activity characteristic of spontaneously active Pl_{HE}.

The physiological significance of multiple transmitters with apparently similar effects on the heart is puzzling. Specificity of action may result from morphological or temporal differences in the domain of transmitter release and, consequently, the particular transmitter(s) and concentrations available to receptors. Differences in morphology of innervation, and activity thresholds do exist for several transmitters in the *Archidoris* heart and may limit activity of particular transmitters to specific regions of the heart. Additionally, simultaneous actions of transmitters, such as the modulatory actions of myomodulin on the *Archidoris* heart, may elicit unique cardiac response. For example, modulatory transmitters acting on gastropod somatic muscles are released in an activity-dependent

manner to change characteristics of the contractile response such as the rate of contraction and relaxation and the magnitude of contraction (Weiss et al., 1992). Finally, the primary actions of some of these transmitters might be to alter other parameters of cardiovascular function such as valve contraction (Lloyd et al., 1985) or filtration characteristics of the myocardium (Skelton and Koester, 1991).

In summary, although the motor component of the cardiac circuit in *Archidoris* appears relatively simple, neurochemical regulation of the heart is complex. Many transmitters, including amines and peptides, have actions on the heart. The large number of active transmitters, particularly those with excitatory actions, and the observation that myomodulin potentiates the inotropic response to 5HT suggests that, as in the somatic muscles of gastropods, interactions between simultaneously released transmitters may be an important mechanism to fine-tune cardiac responses during particular behaviors.

Table 2.1 Actions of amine and peptide transmitters on an isolated preparation of the *Archidoris* heart.

Transmitter	Cardiac Response*	Average Threshold	Amplitude EC_{50}	Rate EC_{50}	n
5HT	+	<10 nM	68 ± 20 nM	12 ± 2 nM	4
DA	+	<100 nM	245 ± 45 nM	207 ± 57 nM	3
Glycine	0	--	--	--	6
ACh	-	<10 nM	275 ± 50 nM	195 ± 50 nM	4
SCP_B	+	<10 nM	408 ± 100 nM	348 ± 100 nM	6
FMRFa	+	<20 μM	--	--	6
SP	0	--	--	--	4
Myomodulin	+	<0.8 μM	1 ± 1 nM	8 ± 10 nM	3
R15α1	0	--	--	--	5
R15α2	+	<3 nM	6 ± 0.7 nM	5 ± 2 nM	4

* +, excitatory; -, inhibitory; 0, no response
--, not applicable
EC_{50}s determined from dose-response curves.

Figure 2.1 Cardiac responses to aminergic transmitters. Tension recordings of heart contractions showing representative responses to submaximal concentrations of 5HT (A), DA (B), and ACh (C). 5HT and DA had similar, predominantly inotropic, excitatory actions, and ACh had both chronotropic and inotropic inhibitory actions. Eighty μl samples of transmitters were applied to the lumen of the heart (arrow) under constant flow conditions via a three-way sample injection valve. The concentration of transmitters reaching the tissue was actually less than that applied due to the volume of the heart. Pump flow rate was two times faster for A and C; 260 vs 130 μl/min. Transmitters were washed out of the heart within approximately 2-4 min by continuous perfusion with ASW.

Figure 2.1

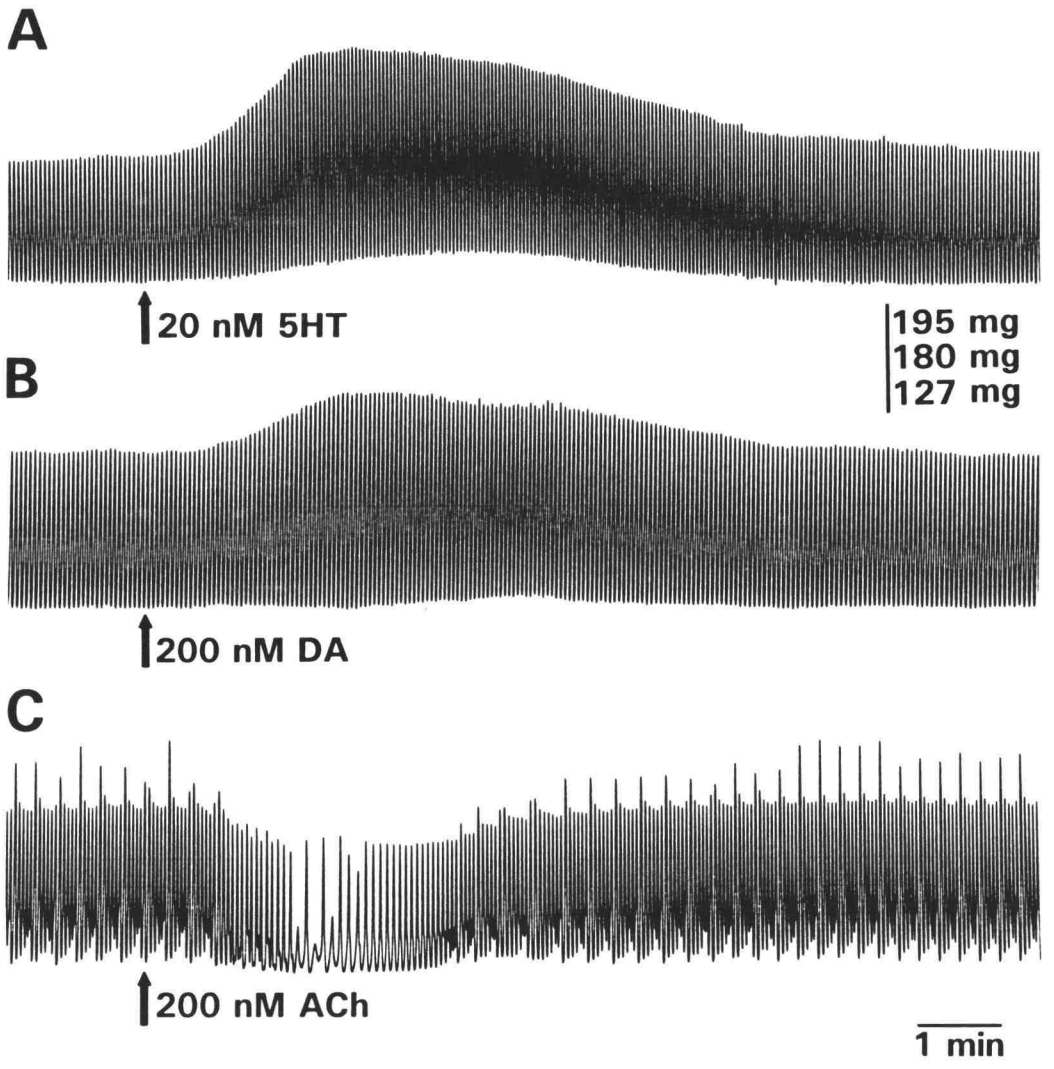
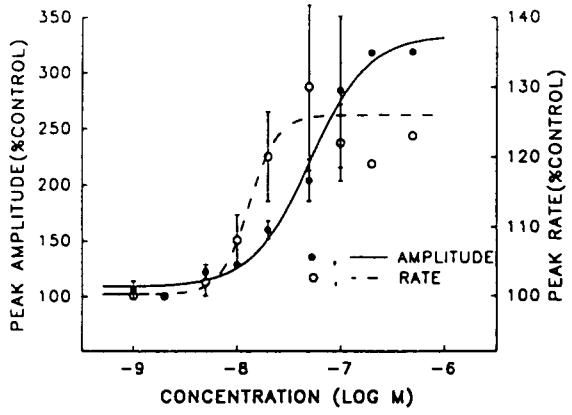


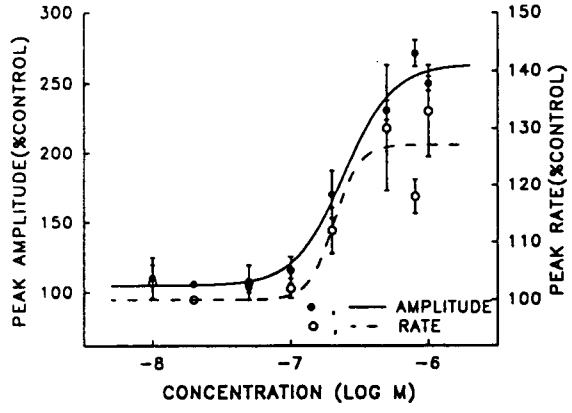
Figure 2.2 Graphical analysis of chronotropic and inotropic responses of isolated hearts to aminergic transmitters. Serotonin (A) and dopamine (B) elicited dose-dependent cardiac responses, maximally affecting the amplitude of contraction at all concentrations. Acetylcholine (C), which decreased the rate and amplitude equally at all concentrations, elicited a dose-dependent inhibitory response. Points are means of normalized peak responses ± SE (A, $n \leq 4$; B, $n \leq 3$; C, $n \leq 4$).

Figure 2.2

A. SEROTONIN



B. DOPAMINE



C. ACETYLCHOLINE

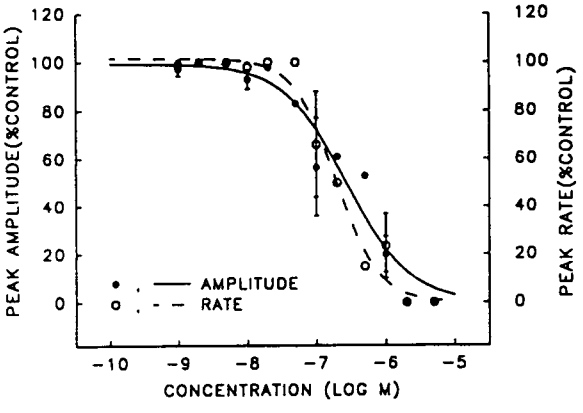


Figure 2.3 Cardiac responses to peptide transmitters. Tension recordings of isolated heart responses to submaximal concentrations of R15α2 (A), SCP_B (B), and myomodulin (C) applied to lumen of heart at arrows. The peptides increased both the rate and amplitude of heart contractions, but at these concentrations the inotropic component of the responses were greater. Pump flow rate was two times faster for A and C; 260 vs 130 μl/min.

Figure 2.3

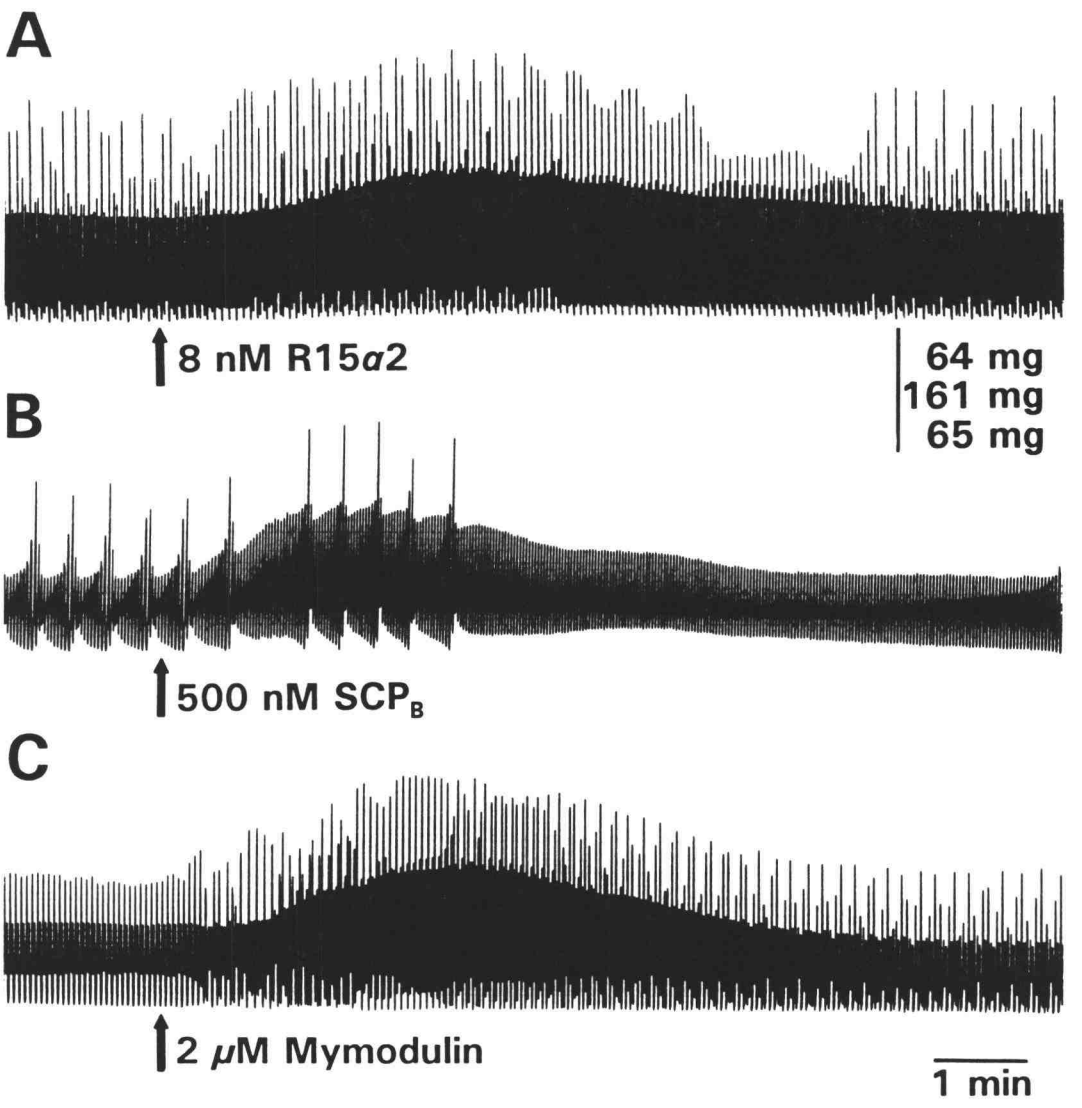
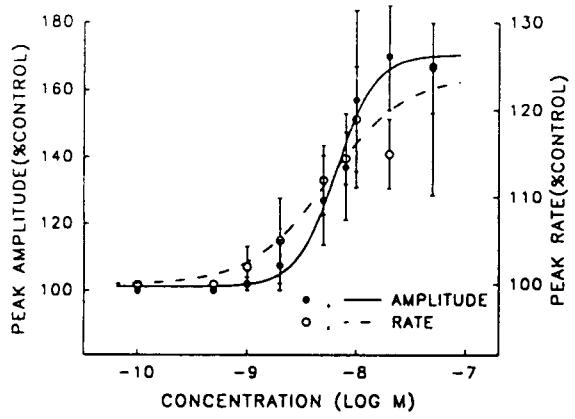
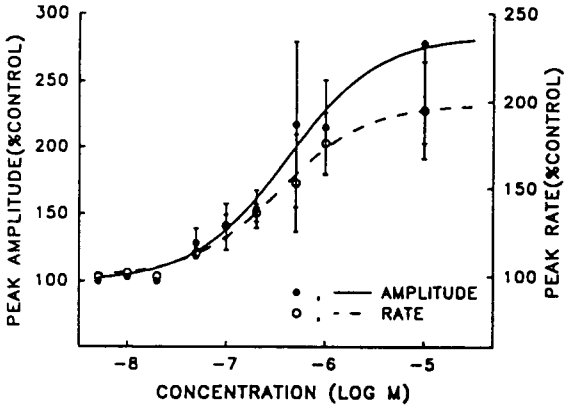


Figure 2.4 Graphical analysis of chronotropic and inotropic responses of isolated hearts to peptide transmitters. R15α2 (A), the most potent excitatory transmitter, had chronotropic and inotropic effects over a relatively narrow concentration range, but at all concentrations inotropic effects predominated. SCP_B (B), also maximally affected contraction amplitude, but the discrepancy between the inotropic and chronotropic effects was not as great. Myomodulin (C) was the only transmitter that appeared to exhibit maximal effects on the rate of contraction, although saturating concentrations for this response were not reached. Points are means of normalized peak responses ± SE (A, $n \leq 4$; B, $n \leq 6$; C, $n \leq 3$).

A. R15a2



B. SCP



C. MYOMODULIN

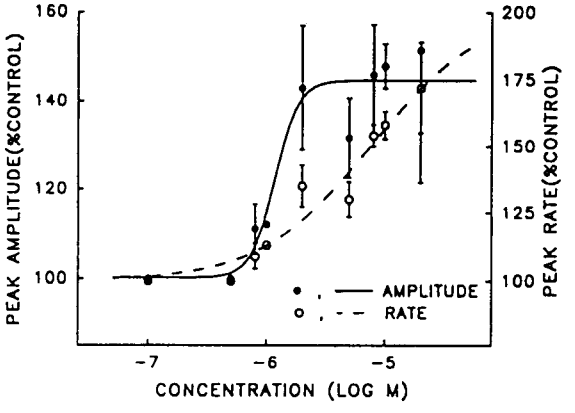


Figure 2.5 Cardio-excitatory actions of FMRFa. Tension recordings of isolated heart contractions show that the excitatory chronotropic, inotropic, and tonotropic cardiac responses increased with ascending concentrations of FMRFa applied at the arrows. Similar to the cardiac response to most of the other excitatory transmitters, the inotropic component of the response to FMRFa was greater at all concentrations tested.

Figure 2.5

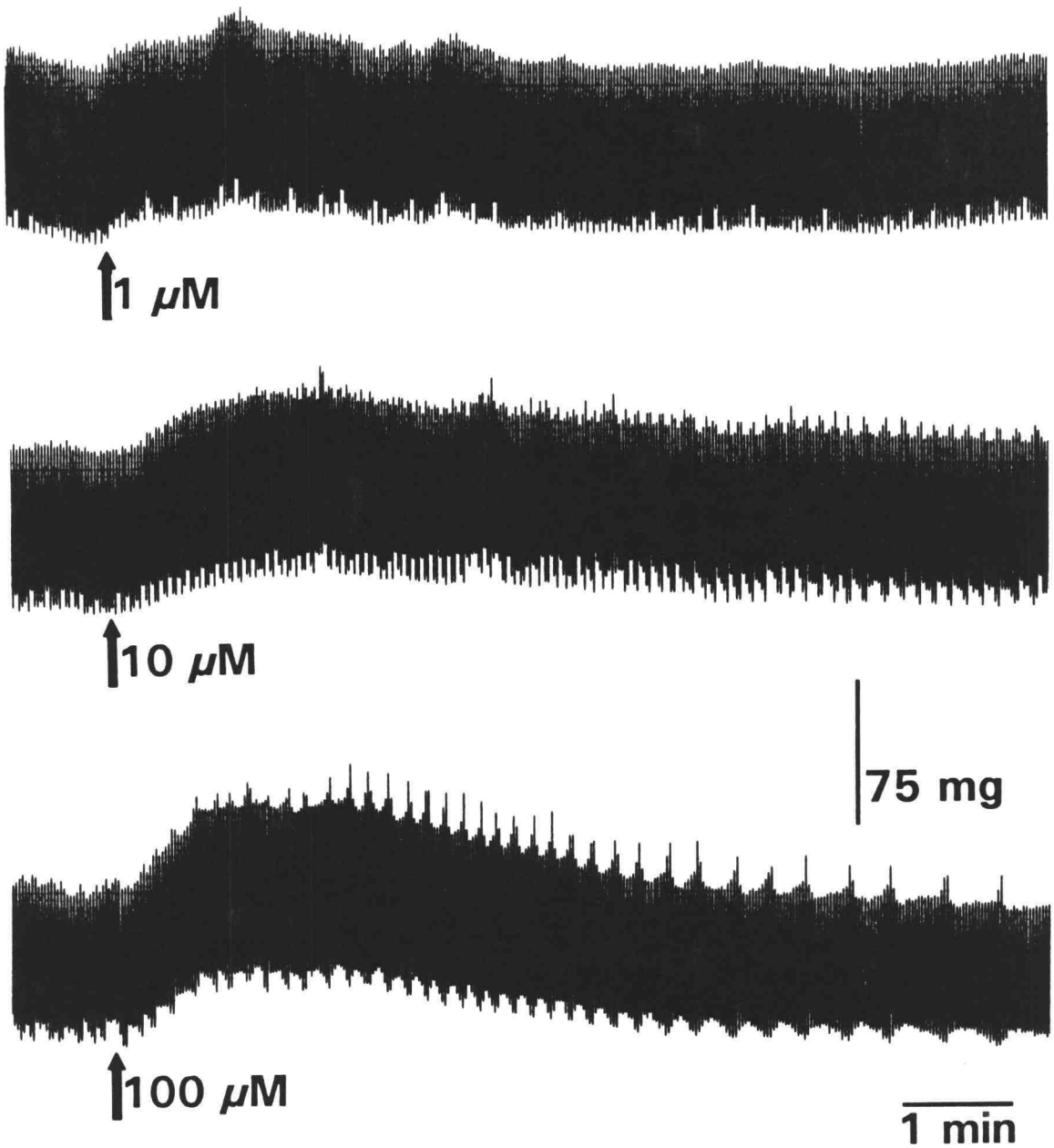
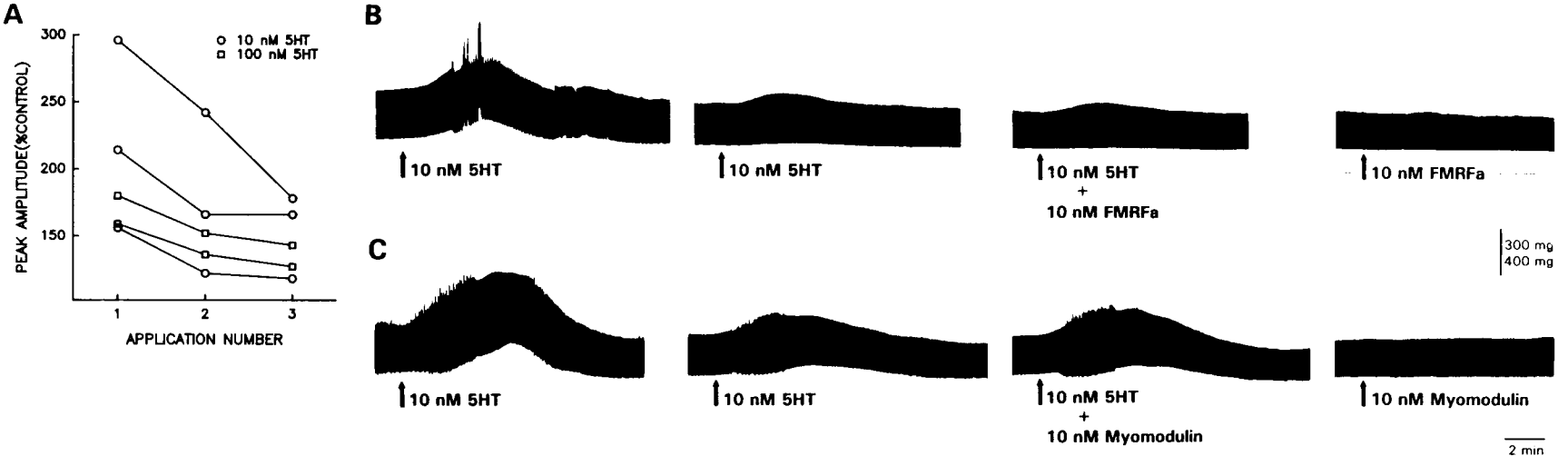


Figure 2.6 Myomodulin modulation of the cardiac response to 5HT. A. Graph showing that the inotropic component of the cardiac response desensitized to multiple applications of 10 or 100 nM 5HT. B. Tension recordings of isolated heart contractions show that the inotropic response desensitization due to repeated application of a submaximal concentration of 5HT (10 nM) was not affected by a subthreshold (10 nM) concentration of FMRFa. C. Inotropic response desensitization was reversed by a subthreshold concentration of myomodulin (10 nM).

Figure 2.6


A
300
250
200
150
PEAK AMPLITUDE(%CONTROL)
1
2
3
APPLICATION NUMBER
10 nM 5HT
100 nM 5HT
B
10 nM 5HT
10 nM 5HT
10 nM 5HT
+
10 nM FMRFa
10 nM FMRFa
C
300 mg
400 mg
10 nM 5HT
10 nM 5HT
10 nM 5HT
+
10 nM Myomodulin
10 nM Myomodulin
2 min

CHAPTER 3

DISTRIBUTION OF CARDIOACTIVE TRANSMITTERS IN THE CENTRAL NERVOUS SYSTEM AND HEART OF THE NUDIBRANCH *ARCHIDORIS MONTEREYENSIS*

ABSTRACT

The heart of the nudibranch *Archidoris montereyensis* is a particularly tractable preparation for investigating the functional significance of multiple transmitter interactions on a single target tissue. Many molluscan cardioactive transmitters, including amines and peptides, are active on the heart of *Archidoris*. The purpose of this study was to determine whether these transmitters are contained within identified cardiac motor neurons or other axonal processes innervating the heart. We show that the excitatory amines, serotonin and dopamine, are likely to be endogenous cardioactive transmitters based on immunocytochemical and biochemical detection of these transmitters in the central nervous system (CNS) and cardiac nerves. The excitatory peptides R15α2, small cardioactive peptide B, myomodulin, and FMRFamide were also detected immunocytochemically in the CNS and nerves innervating the myocardium. The specific distribution of each transmitter within the heart suggests differences in the function of these transmitters that are

consistent with their pharmacological actions on the heart. However, none of these transmitters, including the inhibitory transmitter acetylcholine, were detected in the two motor neurons of the pleural ganglion that most powerfully affect cardiac activities. Thus, the transmitter systems described here may function in some capacity other than short term excitation or inhibition of the heart.

INTRODUCTION

Invertebrate muscle preparations, such as the gastropod heart, are useful model systems in which to investigate the functional significance of multiple transmitter innervation of a single target. A large number of transmitters, including amino acids, amines and peptides, are active on the heart (Walker, 1986), and many appear to be endogenous mediators of neuronal actions. For example, in the opisthobranch *Aplysia californica* 5HT and ACh are synthesized in the excitatory (RB_{HE} and LD_{HE}) and inhibitory (LD_{HI}) cardiac motor neurons, respectively, and the effects of motor neuron stimulation are mimicked by these transmitters and blocked by the appropriate antagonists (Liebeswar et al., 1975). Recently, Skelton and co-workers (1990) showed that the peptide R15α2 is also synthesized by RB_{HE}. Amino acid and oligopeptide transmitters have also been detected (immunocytochemically or biochemically) in other heart regulatory neurons (McAdoo et al., 1978; Price et al., 1978), axonal

terminals within the heart (Harris and Ono, 1990; Skelton and Koester, 1991), or both (Kreiner et al., 1984; Alevizos et al., 1991a), raising the possibility that several transmitter substances participate in regulating activities of the heart.

Our previous work suggests that the heart of the nudibranch mollusc *Archidoris montereyensis* is an advantageous preparation in which to investigate the functional significance of transmitter diversity in the heart. The contractile activity of the heart continues for many hours in dissected preparations of this animal and the cardiac motor neurons maintain a strong influence over this activity. The heart is regulated by five motor neurons; two of these neurons, an excitor and inhibitor in the right pleural ganglion appear to be the major effectors of cardiac activity and are the most potent cardiac motor neurons yet described in molluscs (Wiens and Brownell, 1990a). Despite this relatively simple and effective circuit, transmitter systems regulating the heart are potentially complex (Chap. 2). As in most gastropods, serotonin (5HT) and dopamine (DA) increase the rate and amplitude of contractions, while acetylcholine (ACh) decreases these parameters. A unique subset of molluscan cardioactive peptides - small cardioactive peptide B (SCP_B), FMRFamide (FMRFa), myomodulin and R15α2 - all have excitatory actions on the heart and at least one of these also has indirect modulatory actions. Although the excitatory transmitters have different potencies, all except myomodulin

have actions similar to the heart excitatory motor neurons in that they predominantly affect the amplitude of contraction. Similarly, the actions of ACh, the only inhibitory transmitter, are comparable to those of the inhibitory heart motor neurons in that rate and amplitude of contractions are affected equally.

The objective of this study was to determine whether these cardioactive transmitters are present in neurons and nerves innervating the heart of *Archidoris* and, if so, how their distribution correlates with their apparent functions. Preliminary reports of some of these findings have appeared (Wiens and Brownell, 1990b and 1991)

MATERIALS AND METHODS

Animals: *Archidoris montereyensis* were collected and maintained in a re-circulating, natural seawater aquarium as described previously (Chap. 2).

Immunocytochemistry:

(i) Intact tissues. Methods for immunostaining intact tissues were based on those of Longley and Longley (1986). The circumesophageal, buccal and gastro-esophageal ganglia, heart, and anterior aorta were dissected from animals anesthetized by hemocoel injection of isotonic $MgCl_2$ (approximately 20% of body weight). Dissected tissues were pinned in Sylgard (Dow Corning Corp.) lined petri dishes, rinsed thoroughly with seawater, and incubated in 0.5% protease (Type IV,

Sigma Chemical Co.) for 45-60 min. at room temperature with agitation. Tissues were fixed overnight at 4°C in 4% paraformaldehyde phosphate buffered saline (PBS; 0.1M phosphate buffer with 0.9% NaCl, pH 6.5) and then washed for 3-5 hrs. in PBS. During the wash the connective tissue sheath was dissected from the ganglia. To facilitate antibody penetration, the tissues were dehydrated by incubation in an ascending ethanol series (50% to 100%), followed by 100% methanol, then rehydrated in a descending ethanol series, and washed 1 hr. in 20 mM PBS containing 0.3% Triton X-100 and 0.1% sodium azide (PBS-TA). The alcohol and subsequent incubations were done at 4°C with slight agitation. To block non-specific staining, the tissues were incubated for 12-24 hrs. in PBS-TA with 6% normal goat serum (NGS) and all antisera were diluted in this solution. After blocking, the tissues were incubated in the primary antiserum for 72 hrs., washed in 6% NGS PBS-TA for 24 hrs., incubated in the secondary antiserum (fluorescein or rhodamine conjugated) for 36 hrs, and washed in PBS-TA for 12 hrs., followed by PBS for an additional 6-12 hrs. The tissues were then mounted in 1:9 50mM Tris buffer (pH 9.5)/glycerol with 4% n-propyl gallate (Giloh and Sedat, 1982) and viewed with an epifluorescence microscope (Zeiss standard; with excitation and barrier filters appropriate for fluorescein or rhodamine).

(ii) Sectioned tissues. Ganglia were dissected and fixed as described above, except those to be stained with ACh antiserum which were fixed in 0.1% glutaraldehyde in Bouin's fixative 12 hrs. After fixation, tissues were washed from 3-5 hrs. in PBS and incubated in 20% sucrose PBS until they sank (approximately 1 hr.). The ganglia were then frozen in O.C.T. compound (Miles Scientific, Inc.), cryostat sectioned (20 μm), and mounted on chrom-alum coated glass slides. The sections were incubated for 10 min. in 20 mM PBS-TA, 20 min. in 6% NGS PBS-TA, and then for 12 hrs at 4°C with each primary antiserum except anti-ACh which was left on the tissue for 48 hrs. Sections were then washed for 10 min in PBS-TA (2X), incubated for 1 hr. at room temperature with the secondary antiserum, washed for 10 min. in PBS (2X), and mounted and examined as described above.

(iii) Antisera. The following antisera and dilutions were used: 5HT antiserum (INCSTAR), 1:200; 5HT antiserum, 1:200 and acetylcholine antiserum, 1:500 (Soinila and Mpitsos, 1991); SCP_B monoclonal antibody (Masinovsky et al., 1988), 1:20; FMRFa antiserum 231 (O'Donohue et al., 1984), 1:250; FMRFa antiserum L135 (Williams and Dockray, 1983), 1:250; myomodulin antiserum (Miller et al.,1991a), 1:200; R15 antiserum I/II (Alevizos et al., 1991a), 1:50; Fluorescein (Cappel, 1:200) and rhodamine (Hyclone, 1:20) conjugated goat antirabbit and antimouse antisera.

Specificity of antisera staining was tested by incubating the tissues with blocking solution alone or each antiserum (serotonin, INCSTAR; SCP_B; FMRFa antiserum, O'Donahue; myomodulin; R15 I/II) preabsorbed for 24 hrs. with the appropriate antigen at a concentration of 100 μM (serotonin hydrochloride, Sigma; FMRFa, myomodulin, and SCP_B, Peninsula) or 1μM R15 peptides (gift from K. Weiss). Specificity of ACh staining was confirmed by staining alternate sections with a control antiserum made to glutaraldehyde-hemocyanin. No staining was seen in tissues incubated in blocking solution or in serotonin, SCP_B, or myomodulin pre-absorbed antiserum. Similarly, ACh control antiserum did not stain tissues. FMRFa-absorbed antiserum stained a few cell bodies in the buccal ganglion but did not stain cells in the circumesophageal ganglia or the heart. R15 peptide staining was diminished but not completely blocked by antigen-absorbed antiserum presumably because the 100μM solution of R15α2 peptide we had available to block the antiserum was inadequate. Both of the antisera to 5HT and FMRFa stained the same subsets of neurons and cardiac nerves, further confirming the specificity of staining for these transmitters. An immunocytochemical study such as this, however, has the inherent caveat that the antisera used may not be recognizing exactly the same molecules or peptides to which they were raised. In particular, the peptide antisera, which were made to peptides that belong to intra- and inter-specific families (Mahon et al., 1985b;

Schaefer et al., 1985; Lloyd, 1986; Buck et al, 1987; Price and Greenburg, 1989; Kobayashi and Muneoka, 1990; Bogerd et al., 1991; Miller et al., 1991b) are capable of recognizing other peptides within a family (Dockray et al., 1983; Bulloch et al., 1988; Masinovsky et al., 1988; Miller at al., 1990a; Alevizos et al., 1991a).

Identification of Heart Motor Neurons: We identified heart motor neurons for immunostaining or biochemical analysis (below) by their ability to alter cardiac activity in a semi-intact preparation when intracellularly stimulated through glass micropipettes using standard electrophysiological techniques. Following physiological identification of a heart motor neuron for immunostaining it was iontophoretically injected (hyperpolarizing current pulses of 5 - 10nA , 1 s duration, 0.5 Hz) with a 3% solution of Lucifer Yellow in 0.1% $LiCl_2$ and the ganglia were processed for whole-mount immunocytochemistry using a rhodamine conjugated secondary antiserum as described above. Heart motor neurons to be biochemically assayed were iontophoretically injected as described above with a 5% solution of Fast Green and individually dissected after first softening the sheath with trypsin (2mg/ml Type III, Sigma) for approximately 50 min followed by trypsin inhibitor (2 mg/ml) for 10 min.

Biochemical detection of biogenic amines:

Tissue concentrations of biogenic amines were determined by electrochemical detection of chromatographically separated tissue

constituents in collaboration with N. Syed at the U. of Calgary Medical School. Freshly dissected pleural heart excitatory motor neurons, circumesophageal ganglia, hearts (2/sample), or buccal/gastro-esophageal ganglia (3/sample) were frozen (-70°C) in ASW until analysis. Prior to analysis tissues were thawed and placed into 10 μl of 0.2M pentafluoropropionic acid (PFPA), passed through two freeze thaw cycles, homogenized, and diluted to 100 μl PFPA in an autosampler microvial. Ninety μl samples were applied to a 45 x 4.6 mm Ultrasphere (5 μm) C-18 column (Beckman) using a Waters WISP 710B autosampler. The mobile phase consisted of 30 mM trisodium citrate, 10 mM citric acid, 1 mM EDTA, 100 mM sodium perchlorate, and 10 mM SDS pumped at a flow rate of 2 ml/min. by a Waters HPLC pump (6000A). The identity and concentration of biogenic amines was determined by comparing the signals from tissues and external amine standards using a BAS amperometric detector operated at +0.65V. The detector signal was digitized and recorded using the Waters/Dynamic Solutions Maximal data acquisition system. External standards were freshly prepared in 0.2 M PFPA, and the detection limit was 0.05 pmoles of DA or 5HT.

RESULTS

We used two methods to map the distribution of cardioactive transmitters in the heart and CNS; 1) antisera to localize 5HT, ACh, SCP_B, FMRFa, myomodulin, and the R15 peptides, and 2) chromatographic methods to localize DA and confirm the localization of 5HT.

Localization of transmitters in the CNS.

Immunostaining of both whole and sectioned ganglia allowed us to identify groups of immunoreactive neurons as well as their axonal processes in the ganglionic neuropil. As shown in Figures 3.1 and 3.2, immunocytochemical staining for the transmitters 5HT, ACh, SCP_B, FMRFa, myomodulin, and the R15 peptides revealed distinct and predominantly non-overlapping populations of neurons in the CNS.

Serotonin. The circumesophageal ganglia contained many 5HT-ir neurons (Fig.3.1, A; Fig. 3.3, A). The majority of these neurons were located in three clusters in each pedal ganglion. The cluster of cells on the dorsal surfaces of the pedal ganglia contained at least 60 neurons, and the two clusters on the ventral side contained 50-100 cells each. The ventral surface of each cerebral ganglion also contained a fairly large cluster of 25-50 cells. Only a few 5HT-ir neurons were located in the pleural ganglia, and most of these were located in the left hemiganglion. One large neuron (200-250μm) in each ganglion was bilaterally

symmetrical, and the axons from these neurons appeared to project into the lateral pleural nerves. The neuron in the right pleural ganglion also had a process that extended through the visceral ganglion and into each of its exiting nerves, including the pericardial nerve (PN). The position and size of this neuron did not resemble the primary heart excitor motor neurons, PI_{HE}, and labeling PI_{HE} with Lucifer Yellow prior to immunostaining confirmed this distinction (Fig. 3,3A, inset). The visceral ganglion lacked 5HT-ir neurons, but contained many 5HT-ir processes with varicosities. Likewise, the buccal and gastro-esophageal ganglia contained immunoreactive processes originating from the cerebral-buccal connectives but no 5HT-ir cells bodies (Fig. 3.1).

Biochemical detection of amine transmitters by HPLC confirmed the presence of 5HT in the CNS and revealed that DA is also an abundant transmitter. The CNS of *Archidoris* contained levels of both serotonin and dopamine (Table 3.1) comparable to those detected in the nervous systems of other gastropods (McCaman et al., 1979; Sloley et al., 1990). In contrast, 5HT and DA were not detected in the pleural heart excitor motor neuron. Other molluscan neurons known to be mediated by these transmitters have 5HT or DA levels substantially above the 0.05 picomolar detection limit of this assay (Weinreich et al., 1973; Ono and McCaman, 1984).

ACh. The circumesophageal ganglia contained approximately 40

ACh-ir neurons (Fig. 3.1, B). These were dispersed throughout the ganglia. A small group of ACh-ir cells was present on the ventral surface of the cerebral and rhinophore ganglia. The pleural ganglia contained approximately 3-6 ACh-ir neurons each (Fig. 3.3, B1). The positions of two of these neurons in the right pleural ganglion were similar to Pl_{HI}, however, ACh antisera did not stain Lucifer Yellow labeled Pl_{HI} cells (Fig. 3.3, B1, inset). The largest proportion of Ach-ir neurons were located in the visceral ganglion as approximately 6 of the 25-30 cells which comprise this ganglion were ACh-ir (Fig.3.3, B2).

SCP_B. SCP-ir neurons were dispersed throughout the CNS (Fig. 3.2, A). The SCP-ir neurons in the cerebral ganglia consisted of two groups in each hemiganglion (Fig. 3.2, A). The group on the dorsal surface comprised 25-30 cells, while the ventral surface cluster contained about 5 neurons. Most of the SCP-ir neurons in the pleural ganglia were located ventrally with the most distinct group located laterally in the right hemiganglion. Dorsally the pleural ganglia contained approximately three SCP-ir neurons each. The most posteriorly located neuron in each hemiganglion was bilaterally symmetrical. A dense plexus of SCP-ir processes was concentrated around these cells and extended into the lateral pleural nerves (Fig. 3.3, C1). SCP-ir processes in the cerebral and pleural ganglia form dense, organized processes in the neuropil (Fig. 3.3, C1). Processes also extended into the visceral ganglion and the visceral

nerves, including the PN. Labeling of PI_{HE} prior to immunostaining revealed that this neuron was not one of the SCP-ir cells in the right pleural ganglion (Fig. 3.3, C, inset). The buccal and gastro-esophageal ganglia contained approximately 25-30 SCP-ir neurons (Fig. 3.2, A), including the largest neuron in each gastro-esophageal hemiganglion, and the buccal neuropil contained dense SCP-ir processes.

R15 peptides. A limited number of neurons were immunoreactive for the R15 peptides (Fig. 3.2, B). Most of these were located in a strip of cells on the ventral surface of each cerebral ganglion. The visceral ganglion contained approximately three R15-ir neurons (Fig. 3.3, D), and one large cell was also located on the posterior margin of each pedal ganglion.

Myomodulin. Myomodulin-ir neurons were located in all ganglia (Fig. 3.2, C). One distinct group of 20-40 cells was located on the ventral surface of each cerebral ganglion. Two other groups of cells were present in each pedal ganglia, one located medially on the ventral surface and another laterally on the dorsal surface. The neuropil was extensively labeled in each ganglia. The pleural ganglia contained approximately eleven myomodulin-ir neurons. Labeled processes extended from the right pleural ganglion through the visceral ganglion and into the visceral nerves (Fig. 3.3, F). However, none of these immunoreactive neurons in the right pleural ganglion were PI_{HE} (Fig. 3.3, E, inset). Four groups of

neurons were myomodulin-ir in the buccal/gastro-esophageal ganglia, two posterior clusters and 3-4 larger cells on the anterior side of the buccal ganglia and three bilateral cells in each gastro-esophageal hemiganglion (Fig. 3.2, C). The large neurons in the gastro-esophageal ganglia immunoreactive for SCP_B were also myomodulin-ir.

FMRFa. FMRFa-ir neurons were observed in all ganglia (Fig. 3.2, D). Most of these neurons were present on the dorsal surface of the ganglia. The ventral surface of the pedal ganglia contained a few small neurons surrounded by a dense plexus of FMRFa-ir processes. The FMRFa-ir neurons in the cerebral ganglia were divided into two groups of dispersed cells on the dorsal surface. The pleural ganglia contained a cluster of very small cells and less than 10 larger neurons surrounded by dense FMRFa-ir processes which extended into the lateral pleural nerves (Fig. 3.3, F). These cells were in the same region as PI_{HE}, but this neuron, identified by Lucifer Yellow labeling, was not FMRFa-ir (Fig. 3.3, F, inset). The large anterior FMRFa-ir neuron in the right hemiganglion appeared to be homologous to the SCP-ir neuron. FMRFa-ir processes extended into the visceral ganglion and each of the visceral nerves. Within the ganglion these processes contained dense varicosities. Each buccal hemiganglion contained a medial FMRFa-ir neuron (100 μm) and a cluster of approximately 10 smaller neurons (Fig. 3.2, D). The gastro-esophageal ganglia each contained a small group of FMRFa-ir neurons.

Localization of transmitters in the heart.

With the exception of the ACh antiserum, the same antisera used to map the distribution of transmitters in the CNS stained nerves innervating and embedded in muscle tissue of the heart (Figs. 3.4 and 3.5). ACh antisera could not be used on tissues outside of the CNS because it exhibited high levels of non-specific staining. Immunostaining revealed two general patterns of transmitter distribution; 5HT, SCP_B, and R15α2 antisera stained nerves throughout the ventricular and atrial myocardia, while FMRFa and myomodulin antisera labeled nerves in more localized regions.

Serotonin. 5HT-ir processes entered the heart through both the arterial and ventricular branches of the PN (Fig. 3.4, A). Processes from these branches broadly innervated the ventricular and atrial myocardium (Fig. 3.5, A1). The AV valve was also densely innervated with processes containing many varicosities (Fig. 3.5, A2). Additional 5HT-ir processes also innervated the atrium via the lateral pleural nerve (RPln4). The lumenal wall of the anterior aorta possessed a sparse 5HT-ir network. The presence of 5HT in the heart was confirmed by electrochemical detection of HPLC fractions of heart extracts and similar levels of DA were also detected (Table 3.1).

SCP_B. SCP_B-ir processes innervated the ventricular (Fig. 3.5, B) and atrial myocardium via the arterial and ventricular branches of the

pericardial nerve (Fig. 3.4, B). The distribution of these processes was very similar to that of 5HT-ir processes but much less dense. SCP_B and 5HT double-labeling immunocytochemistry revealed that the processes immunoreactive for these transmitters are distinct.

R15 peptides. The distribution and density of R15-ir processes in the heart was similar to that of SCP_B-ir processes (Fig. 3.4, C). R15-ir processes innervated the myocardium of the ventricle and atrium (Fig. 3.5, C1) and the AV valve via the arterial and ventricular branches of the PN. Small cell bodies in the atrium were R15-ir (Fig. 3.5, C2).

Myomodulin. Myomodulin-ir in the heart was limited to the anterior portion of the ventricle innervated via the arterial and ventricular branches of the PN (Fig. 3.5, D) and a few immunoreactive processes on the efferent gill vein (Fig. 3.4, D). A sparse immunoreactive network was also present in the anterior aorta.

FMRFa. FMRFa-ir processes sparsely innervated the ventricular and atrial myocardium via the arterial and ventricular branches of the PN and RPIn4 (Fig. 3.4, E). The most pronounced FMRFa-ir was on the aortic valve (Fig. 3.5, E), the anterior aorta, and the efferent gill vein, which were densely innervated with FMRFa-ir processes. A group of small cell bodies on the lumenal wall of the atrium were also FMRFa-ir (Fig. 3.5, F). These were in the same location as the R15 peptide-ir cells, but we were unable to determine if they were the same cells.

DISCUSSION

In this study we used immunocytochemical and biochemical techniques to map the distribution of cardioactive transmitters in the CNS and heart of the nudibranch *Archidoris montereyensis*. Our results indicate that although the transmitters 5HT, DA, ACh, SCP_B, R15α2, myomodulin, and FMRFa were not present in the identified cardiac motor neurons of the pleural ganglion, they were present in the CNS and nerves innervating the heart. The distribution of these transmitters, in conjunction with their specific activity on isolated preparations of the heart (Chap.2), suggests that they mediate neural regulation of the cardiovascular system in *Archidoris.*

Distinct populations of immunoreactive neurons for the excitatory transmitters 5HT, SCP_B, R15α2, myomodulin, and FMRFa were observed in the CNS. Of these neurons, 5HT, SCP_B, myomodulin, and FMRFa-ir neurons were located posteriorly in the right pleural ganglion as was the heart excitor, PI_{HE}. Similarly, ACh-ir neurons were located in the same region of this ganglion, which also contained the heart inhibitor, PI_{HI}. The absence of immunoreactivity for any of these transmitters in pleural heart motor neurons and the lack of biochemically detectable 5HT or DA in these neurons, however, suggests that other transmitters mediate the actions of these cells. Alternatively, it is possible that the transmitters were not present in high enough concentrations in the soma of the heart

motor neurons to be immunocytochemically detected (Storm-Mathiessen and Ottersen, 1986). This is unlikely to be the case for 5HT in the heart excitor, PI_{HE}, because the immunocytochemical data were confirmed by biochemical analysis capable of detecting catecholamine concentrations down to sub-picomolar concentrations. The immunocytochemical results for the other transmitters must be confirmed with biochemical and/or pharmacological analysis of transmitter identity, before we can conclude that the other transmitters are absent from motor neurons, especially ACh whose actions mimic those of the inhibitory heart motor neuron.

Two of the transmitters, ACh and R15α2, may mediate the actions of the visceral heart motor neurons as neurons immunoreactive for these transmitters were located in this ganglion. In this regard, it is noteworthy that both of these transmitters are synthesized by cardiac motor neurons in the abdominal ganglion of *Aplysia* whose locations and actions appear to be analogous to the visceral motor neurons in *Archidoris* (Mayeri et al., 1974; Wiens and Brownell, 1990a).

Despite their absence in the identified cardiac motor neurons of the pleural ganglion, the transmitters 5HT, SCP_B, R15α2, myomodulin, and FMRFa are involved in neuronal regulation of the heart since nerves innervating the heart are immunoreactive for these excitatory transmitters. These transmitters may mediate the actions of unidentified cardiac motor or neurosecretory neurons. The latter possibility seems more likely given

that we previously conducted an extensive search for cardiac motor neurons, and activity in those identified, particularly the pleural neurons, appears to account for most changes in cardiac activity observed in dissected preparations of the animal. The right pleural or visceral ganglia are the most likely ganglia to contain such neurons because $CoCl_2$ backfills of the pericardial nerve innervating the heart predominantly labeled neurons in these ganglia. We did observe 5HT, SCP_B, myomodulin, and FMRFa-ir neurons in the right pleural ganglion with processes that extend through the visceral ganglion and into the pericardial nerve. The large 5HT-ir cell was identifiable in semi-intact preparations of *Archidoris*, but stimulation of this neuron did not cause a change in the rate, amplitude, or tonus of heart contractions. Thus, this neuron either does not innervate the heart or it may act to alter other parameters of cardiac function analogous to actions of the metacerebral giant cell in *Aplysia* (Lloyd et al., 1984). This serotonergic neuron does not act directly on the accessory radula closer muscle which it innervates, but potentiates the contractile response of the muscle to ACh, the transmitter mediating the excitatory actions of motor neurons.

The distinct distributions of nerves immunoreactive for the excitatory transmitters in the heart suggest they have unique cardioregulatory functions. The distribution of 5HT, SCP_B, and R15 peptide-ir nerves on the A-V valve, the probable location of the cardiac

pacemaker, and throughout the myocardium is consistent with the observation that moderate to low concentrations of these transmitters affected both the rate and amplitude of heart contractions. The specific physiological function of each transmitter remains unclear, however, since they have similar pharmacological actions on isolated hearts and apparently overlapping release sites. In contrast, nerves immunoreactive for FMRFa and myomodulin innervated more localized regions of the heart. The high concentration of these transmitters required to affect rate and amplitude of contraction in isolated hearts indicates that FMRFa and myomodulin may be released locally to alter other parameters of cardiac function. For example, the dense aggregation of myomodulin-ir processes on the ventricular myocardium may be the site at which this transmitter acts on myocytes to potentiate their response to 5HT. The function of FMRFa is less clear, but FMRFa innervation of the aortic valve may regulate its contraction in the same manner as transmitters control contraction of the AV valve of the *Aplysia* heart *in vivo* (Lloyd et al., 1985). Alternatively, the concentration of FMRFa-ir terminals in the aortic valve and efferent gill vein could be neurohemal release sites similar to those described ultrastructurally in the heart of the pulmonate *Helix pomatia* (Cottrell and Osborne, 1969).

The immunocytochemical experiments described also revealed other characteristics of cardiac innervation in *Archidoris*. First,

immunostaining of 5HT and FMRFa-ir processes in the heart showed a previously unidentified source of cardiac innervation entering at the base of the atrium. The nerve RPl_{N4}, which increases tonus of heart contractions when stimulated, appears to terminate in this region of the heart after entering and re-emerging from the posterior body wall. Previously, we attributed this response to a peripheral nerve network due to its latency (Wiens and Brownell, 1990a), but the immunological staining of this nerve shows that neural innervation of the heart is via RPl_{N4} as well as the pericardial nerve. Secondly, these studies revealed a small number of FMRFa and R15 peptide immunoreactive cells in the atrium near its junction with the efferent gill vein. We could not determine whether antisera to these peptides labeled identical or distinct cells. Similar FMRFa immunoreactive cells were also recently observed in the atrium of *Aplysia* (Harris and Ono, 1990). The function of the cells is unknown, but they appear to be neurons judging from their size and associated processes.

Table 3.1 Amine concentrations in the CNS, PI_{HE}, and heart.

TISSUE	SEROTONIN pmoles/tissue*	DOPAMINE pmoles/tissue*	n
CNS			
Circumesophageal	1247.7 ± 70.4	1103.0 ± 173	7
Buccal/Gastro-esophageal	6.1	13.0	2
PIHE	ND	ND	5
HEART	1.1 ± 0.3	1.0 ± 0.6	3

*Values represent average concentrations of amines ± SEM for $n>3$.
Each sample contained the following numbers of tissues:
1 circumesophageal ganglion; 3 buccal/gastro-esophageal ganglia;
1 neuron; 2 hearts. ND, not detectable.

Figure 3.1 Schematic diagrams showing the positions of neurons immunoreactive (-ir) for the aminergic transmitters 5HT (A) and ACh (B) in the buccal/gastro-esophageal and circumesophageal ganglia. Diagrams are based on observations made from 8 and 5 ganglia immunostained for 5HT and ACh, respectively. Filled circles, dorsal neurons; open circles, ventral neurons. B, buccal; C, cerebral; GE, gastro-esophageal; Pd, pedal; Pl, pleural; Rh, rhinophore; and V, visceral ganglia. CBC, cerebral buccal connective; PN, pericardial nerve.

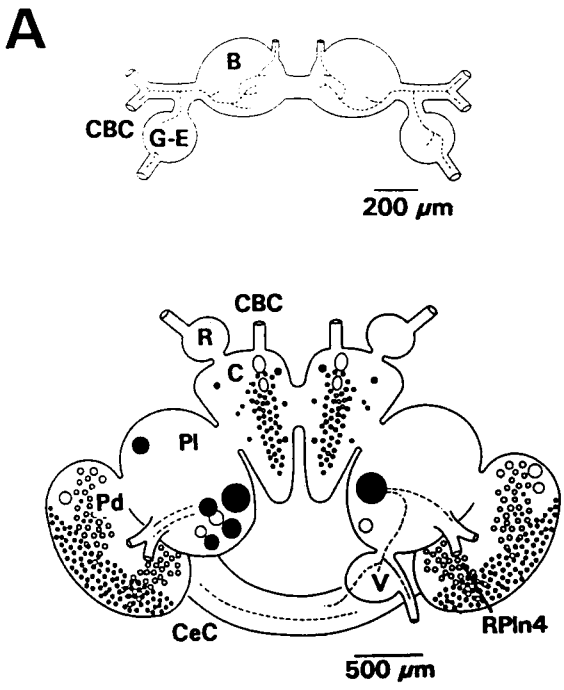

A
B
CBC
G-E
200 μm
CBC
R
C
Pl
Pd
V
CeC
RPln4
500 μm
B

Figure 3.2 Schematic representation of neurons immunoreactive for the peptide transmitters SCP_B (A), R15α2 (B), myomodulin (C), and FMRFa (D) in the buccal/gastro-esophageal and circumesophageal ganglia. Diagrams are based on observations made from 3, 11, 6, and 7 ganglia, respectively. Filled circles, dorsal cells; open circles, ventral cells. Abbreviations the are same as in Fig. 3.1.

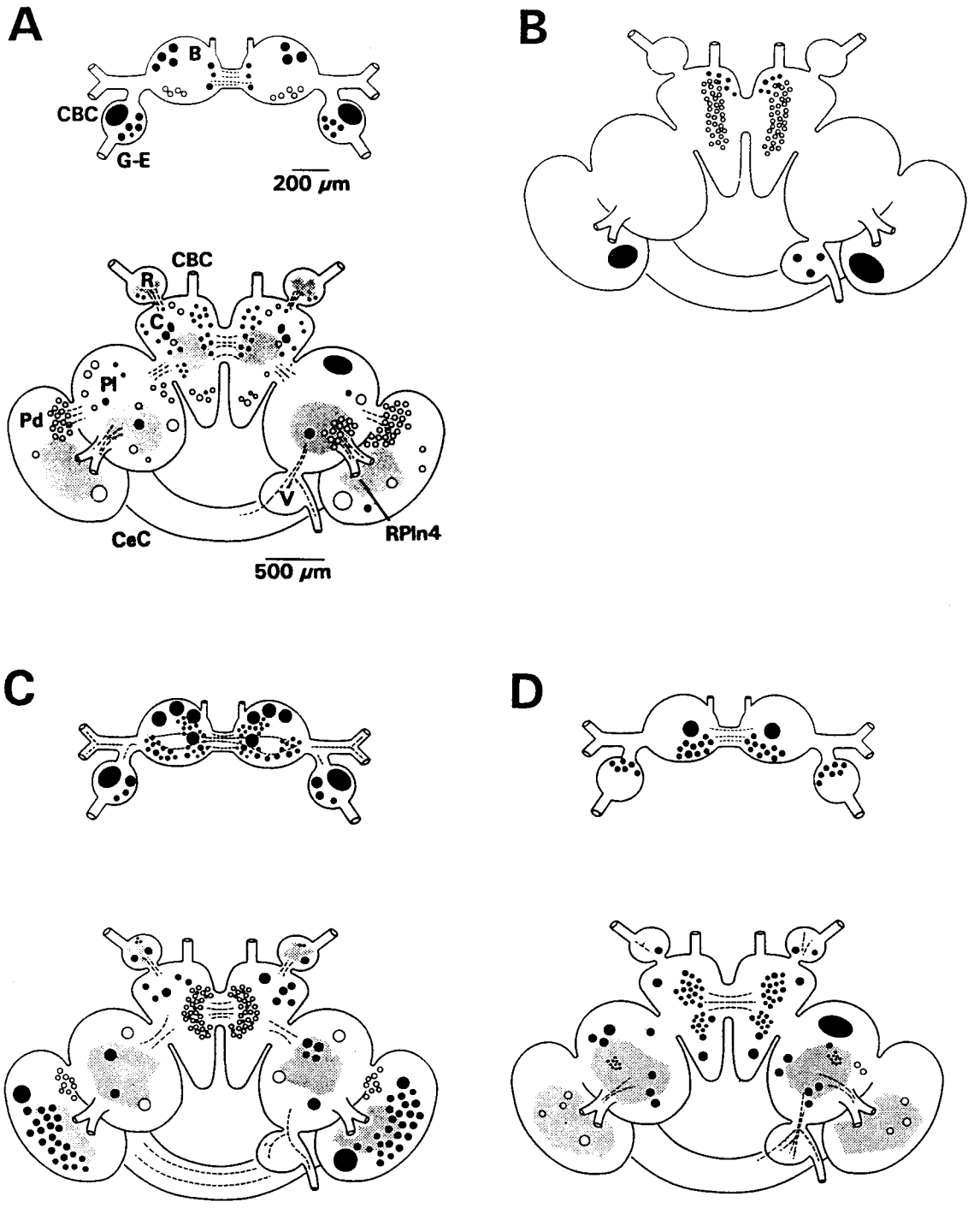

A
B
CBC
G-E
200 μm
CBC
R
C
Pl
Pd
V
CeC
RPln4
500 μm
B
C
D

Figure 3.3 Photomicrographs of transmitter immunostaining in ganglia containing identified heart motor neurons. A. 5HT-ir neurons located dorsally in circumesophageal ganglion. Inset: Pl_{HE} was not 5HT-ir; Lucifer Yellow filled Pl_{HE} photographed with a fluorescein filter set (left); 5HT-ir neurons photographed with a rhodamine filter set (right). B1. An ACh-ir neuron in the right pleural ganglion. Inset: The inhibitory heart motor neuron in this ganglion, Pl_{HI} (left), was not ACh-ir (right). B2. ACh-ir neurons in the visceral ganglion. C1. SCP_B-ir neuron and processes in the right pleural ganglion. Inset: Pl_{HE} (left) was not SCP_B-ir (right). C2. Unique SCP_B-ir structured neuropil in the right pleural ganglion. D. R15 peptide-ir neurons in the visceral ganglion. E. Myomodulin-ir neurons and processes in the right pleural ganglion. Inset: Pl_{HE} (left) was not myomodulin-ir (right). F. FMRFa-ir neurons in the right pleural ganglion with processes extending into the visceral ganglion. Inset: Pl_{HE} (left) was not FMRFa-ir (right). C, cerebral ganglion; Pd, pedal ganglion; Pl, pleural ganglion; V, visceral ganglion. Scale = 50μm.

Figure 3.3

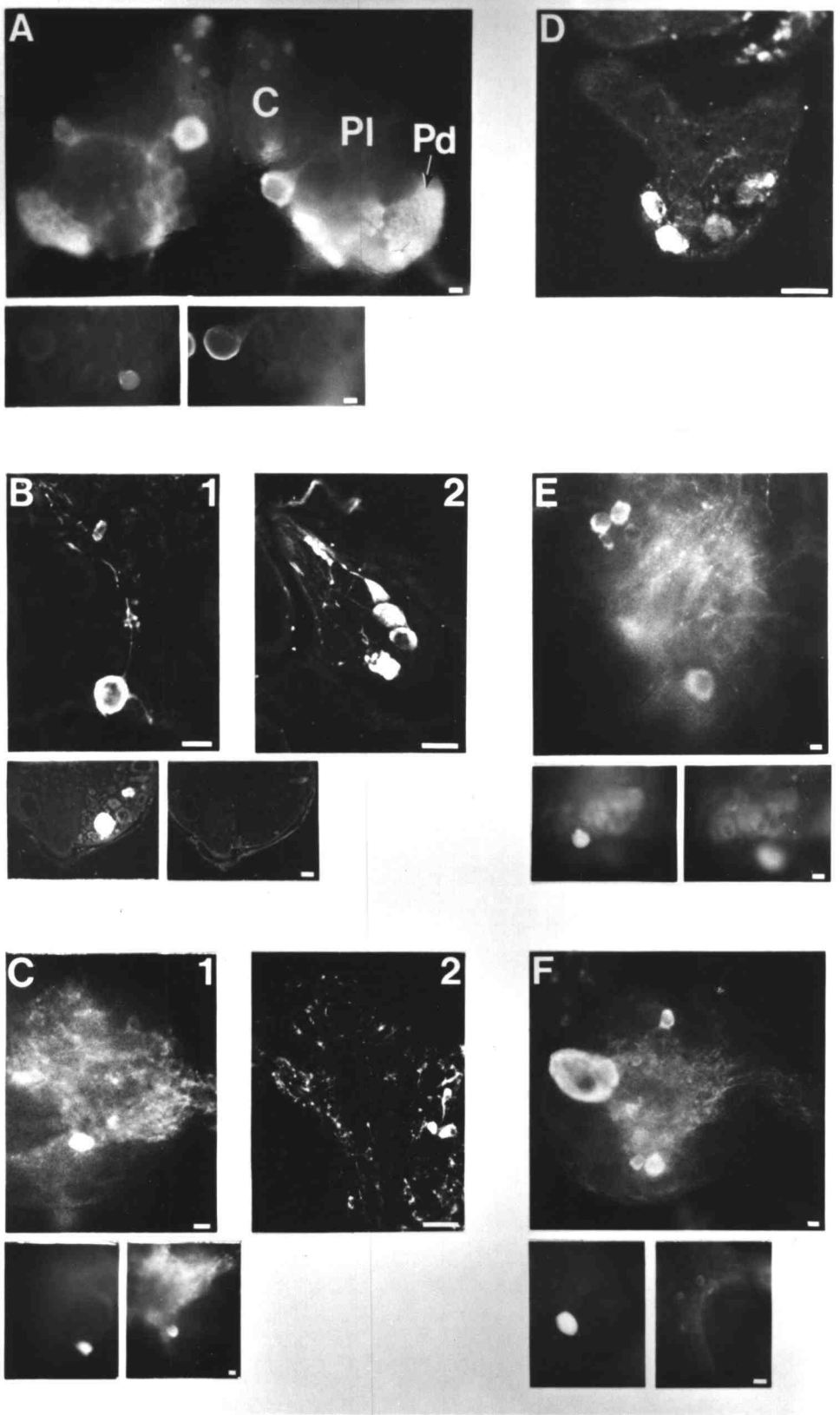


Figure 3.4 Schematic representations of cardiac and aortic nerves immunoreactive for aminergic and peptide transmitters.

A-C. 5HT-ir, R15α2-ir, and SCP_B-ir processes broadly innervated ventricular and atrial myocardium and the A-V valve via the aortic and ventricular branches of the PN. The branch of RPI_{N4} which innervates the atrium also contained serotonin-ir axons. D. Myomodulin-ir processes innervated a limited region of the anterior venticular myocardium via the aortic and ventricular branches of the PN. E. FMRFa-ir processes densely innervated the anterior aorta and the aortic valve via the aortic branch of the PN and the efferent gill vein via an unknown nerve. RPI_{N4} also contained FMRFamide-ir axons. Additionally, a small cluster of cell bodies in the atrium were FMRFamide-ir. Diagrams are based on observations made from 4, 2, 3, and 5 hearts, respectively. AA, anterior aorta; A, aortic valve; At, atrium; A-V, atrioventricular valve; EG, efferent gill vein; PN, pericardial nerve; V, ventricle.

Figure 3.4

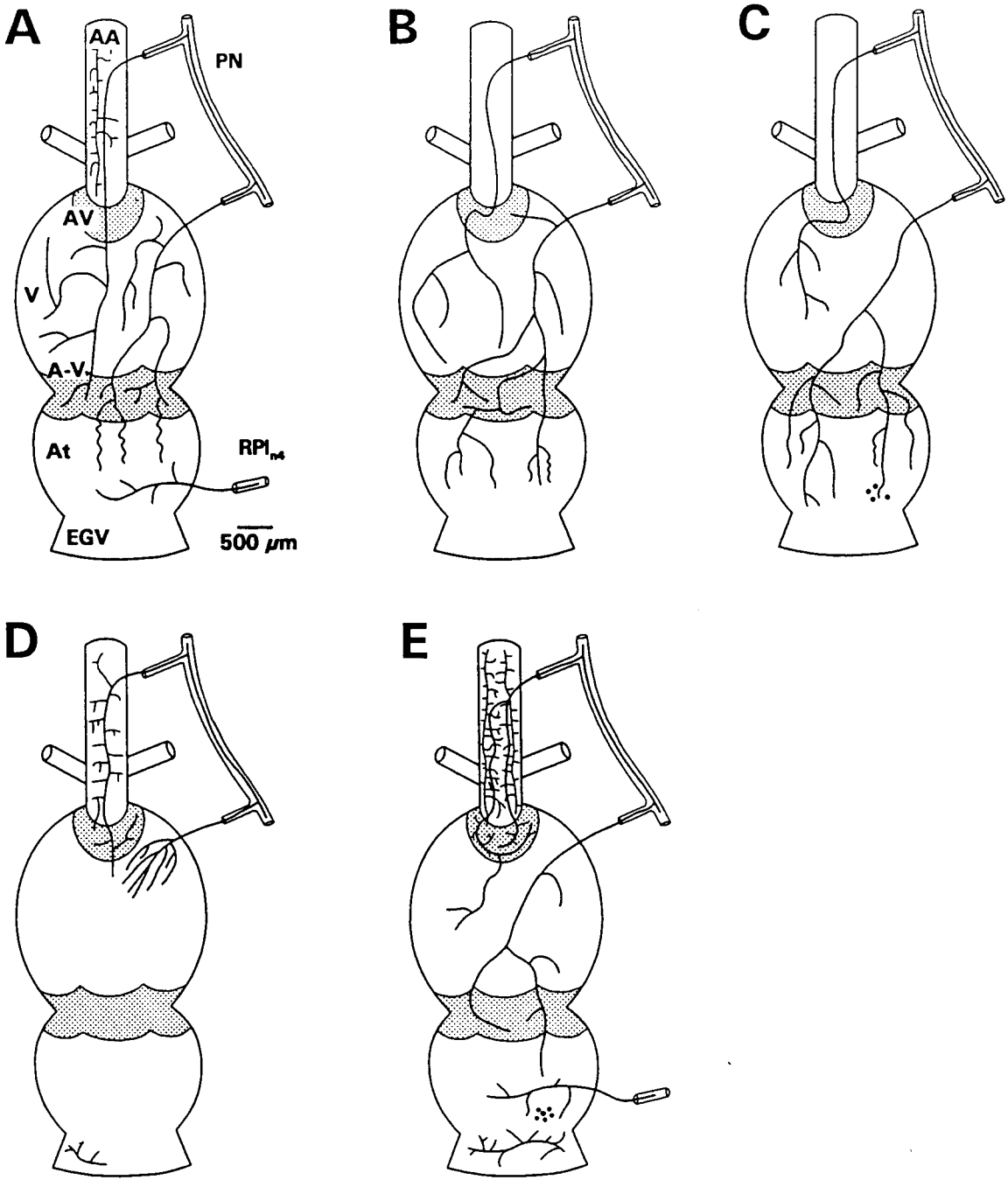
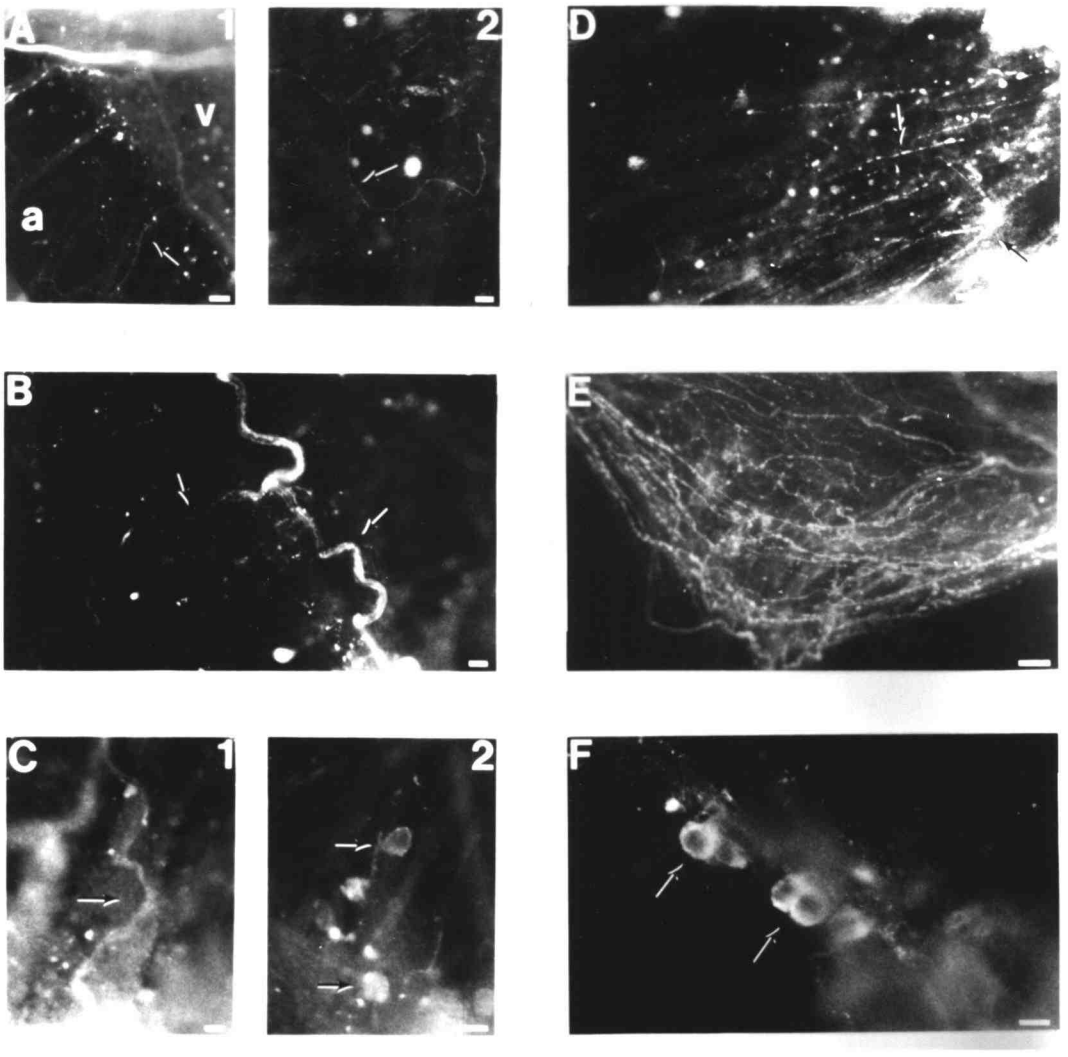


Figure 3.5 Photomicrographs of transmitter immunoreactivity in the heart. A1. 5HT-ir processes (arrow) along atrial trabeculae. A2. 5HT-ir processes with varicosities on the AV valve (arrow). B. SCP_B-ir processes in nerves innervating the ventricular myocardium (arrows). C1. R15 peptide-ir processes (arrow) along muscular trabeculae in the atrium. C2. R15 peptide-ir cell bodies (arrows) in the atrium. D. Concentration of myomodulin-ir processes in the anterior ventricle (arrows). E. Dense FMRFa-ir processes innervating the aortic valve. F. FMRFa-ir cells (arrows) in the atrium. a, atruim; v, ventricle. Scale = 25μm.

Figure 3.5



CHAPTER 4

A NEUROENDOCRINE SYSTEM INDUCING EGG-LAYING BEHAVIOR IN THE NUDIBRANCH *ARCHIDORIS MONTEREYENSIS*

ABSTRACT

We describe a group of neurons with egg-laying bioactivity in the cerebral ganglia of an opisthobranch mollusc, the nudibranch *Archidoris montereyensis*. These cells, the Intercerebral White Cells (IWCs), share morphological, biochemical, and electrophysiological characteristics with the egg-laying neuroendocrine cells of *Aplysia californica* (bag cells) and *Lymnaea stagnalis* (caudodorsal cells). The IWCs were located in two superficial clusters of about 100 neurons each immediately posterior to the intercerebral commissure in the cerebral ganglia. The soma of these cells were small (<20 μm), possessing varicose bifurcating unipolar processes. Collectively the processes formed a ring-like structure in the intercerebral commissure with bilateral extensions into the cerebral ganglia. The IWCs and their processes in the commissure were surrounded by a major vascular sinus of the ganglion, a potential neurohemal release site. Homogenates of the IWC clusters and commissure, or of whole cerebral ganglia, induced egg-laying behavior after injection into the hemocoel of quiescent animals. The IWCs were

immunoreactive for *α*BCP, a peptide encoded by the *Aplysia* egg-laying hormone gene that is conserved with a peptide encoded by the homologous gene in *Lymnaea*. Electrophysiological manipulations of the IWCs showed that these electrically silent neurons with large resting potentials were highly refractory to electrical stimulation. Based on these properties the IWCs of *Archidoris* appear to be neuronal homologs of the egg-laying neuroendocrine cells in *Aplysia* and *Lymnaea*, suggesting that all gastropods have similar neuronal mechanisms for control of this behavior.

INTRODUCTION

The neuroendocrine systems regulating egg-laying behavior in gastropod molluscs are uniquely accessible experimental systems for studying the neuronal basis of long-term behaviors. Egg-laying neurohormones are documented in all gastropod subclasses (prosobranchs: Ram, 1982a; opisthobranchs: Kupfermann, 1967, 1970; Toevs and Brackenbury, 1969; Ram, 1977, 1982b; pulmonates: Geraerts and Bohlken, 1976; Takeda, 1977; Geraerts et al., 1983), but, the neurons producing these hormones have only been extensively characterized in a few gastropods, mainly the opisthobranch *Aplysia californica* and the pulmonate *Lymnaea stagnalis* (for reviews see Geraerts et al., 1988). In these animals egg-laying involves long-term (>1 hour),

stereotyped changes in locomotory and visceromotor behavior that culminates in the deposition of an egg-mass on the substrate. Peptides released from the bag cells (BCs) of *Aplysia* and caudodorsal cells (CDCs) of *Lymnaea* act both within the nervous system and on reproductive organs to produce this behavior.

In addition to their common behavioral actions, the BC and CDC neuroendocrine systems exhibit similar morphological, physiological, and biochemical properties suggesting they are members of a larger family of ovulogenic neurons controlling egg-laying in all gastropods. In both animals, these cells exist in bilateral clusters and possess processes that terminate near vascular spaces within the central nervous system (CNS; Coggeshall, 1966; Vlieger et al., 1980). The BC clusters in the abdominal ganglion *Aplysia* each consist of about 400 multipolar neurons whose processes terminate in the connective tissue sheath of the ganglion and the pleuroabdominal connectives (Coggeshall, 1966; Frazier et al., 1967). The CDCs comprise smaller clusters of 40 (left) and 100 (right) neurons located in the cerebral ganglia. The CDCs are unipolar, and two populations can be distinguished morphologically. The majority of the CDCs possess one axon that terminates in the neurosecretory area of the cerebral commissure. A smaller number of ventral CDCs (approximately 8 cells) may mediate electrical coupling between the CDC clusters as these neurons possess an additional process that crosses the commissure and

loops around the axons of the contralateral cluster (Vlieger et al., 1980).

Functional homology in gastropod ovulogenic neurons is further indicated by their unique electrophysiological and biochemical properties. Electrophysiologically the BCs and CDCs are normally silent cells that fire in a prolonged burst of action potentials (afterdischarge) immediately prior to the initiation of egg-laying (Pinsker and Dudek, 1977; Ter Maat et al., 1986). During this afterdischarge, these neurons, which are electrically coupled, fire in unison releasing peptide transmitters that are products of single genes with homologous base sequences (Kaczmarek et al., 1979; Vlieger et al., 1980; Scheller et al., 1983; Stuart et al., 1980; Geraerts and Hogenes, 1985; Vreugdenhil et al, 1988). At least two of these regions have similar physiological functions. The ovulation hormones, Egg-Laying Hormone (ELH) in *Aplysia* and Caudodorsal Cell Hormone (CDCH) in *Lymnaea*, each have 36 amino acids and exhibit 50% homology in primary structure. Additionally, alpha bag-cell peptide (αBCP) and alpha caudodorsal cell peptide (αCDCP) in *Aplysia* and *Lymnaea*, respectively, share 60% homology and function as transmitters in the central nervous system and putative autoexcitatory transmitters (Rothman et al, 1983a; Mayeri et al., 1985; Ter Matt et al., 1988; Brown and Mayeri, 1989).

The ELH gene and CDCH genes are members of multigene families (Scheller et al., 1983; Mahon et al., 1985a; Nambu and Scheller, 1986;

Van Minnen et al., 1989) whose products are expressed in central and peripheral neurons besides the BCs or CDCs (Chiu and Strumwasser, 1984; Pulst et al., 1988; Van Minnen et al., 1988, 1989) as well as in exocrine cells of the reproductive tract (Van Minnen et al., 1989; Mahon et al., 1985a). Although the function of many of these cells is unknown, those in the CNS are thought to be components of a descending neural pathway that initiates egg-laying behavior. For example, ELH/αBCP immunoreactive neurons in the pleural ganglia of *Aplysia*, with similar electrophysiological properties to the BCs, were shown to trigger BC bursts when intracellularly stimulated (Brown et al., 1989). In contrast, peptides expressed in exocrine cells may act as pheromones inducing mating behavior in other animals (Painter et al, 1989, 1991

The common properties of the BC and CDC ovulogenic neurons suggest that the neuronal mechanisms controlling egg-laying behavior in all molluscs are similar. Putative ovulogenic neurons have been identified in other pulmonates by morphological and immunocytochemical criteria, however, the physiological actions of these neurons have not been demonstrated (Roubos and Van De Ven, 1987; Mukai and Saleuddin, 1989; Khan et al., 1990; Van Minnen et al., 1992). In this paper we characterize neuroendocrine cells that stimulate egg-laying in the nudibranch opisthobranch, *Archidoris montereyensis*. The morphology,

transmitter phenotype, and behavioral actions of these intercerebral white cells (IWCs) are similar to those of the BCs in *Aplysia* and the CDCs in *Lymnaea*.

MATERIALS AND METHODS

Animals: Adult *Archidoris montereyensis* were collected from low rocky intertidal areas along the central Oregon coast. Animals were fed their native food, the sponge Halicondria sp. and maintained in a re-circulating, natural seawater aquarium (13-16°C; 16L/8D photoperiod). Each animal was kept in an individual plastic container so that egg-laying episodes could be monitored prior to and during experiments. Only animals that exhibited normal locomotory activity were used in these experiments. Prior to dissection animals were anesthetized by hemocoel injection of an isotonic $MgCl_2$ solution (315 mM; volume equal to 20% of body weight).

Morphology:

(i) Immunocytochemistry. For immunostaining of intact tissues, circumesophageal ganglia were dissected from anesthetized adult animals, immobilized on a Sylgard (Dow Corning, Corp.) foundation in petri dishes, rinsed in buffered (5 mM Hepes, pH 7.8) artificial seawater (ASW), and incubated in 0.5% protease (Type IV, Sigma Chemical Co.) ASW for 45-60 min. at room temperature to permeabilize the ganglionic sheath. Ganglia were then fixed overnight in 4% paraformaldehyde in 0.1 M

phosphate buffer with 0.9% NaCl, pH 7.4 (PBS), washed for 1-3 hrs. in PBS, and desheathed. Tissues were dehydrated by incubation (10 min. each) in an ascending ethanol series (50, 70, 90, 95, 100%), followed by 100% methanol, then rehydrated in a descending ethanol series, and washed 1 hr. in 20 mM PBS containing 0.3% Triton X-100 and 0.1% sodium azide (PBS-TA). The alcohol and subsequent incubations were done at 4°C with slight agitation. Non-specific staining was blocked by incubating the tissues in PBS-TA containing 6% normal goat serum (NGS) for 12-24 hrs. and by diluting antiserum with this solution. After blocking the tissues were incubated in the primary antiserum (72 hrs), 1:10 rabbit anti-αBCP 1-9 (compliments of E. Mayeri, UCSF), washed (12 hrs.) in 6% NGS PBS-TA, incubated in the secondary antiserum (36 hrs.), 1:100 goat anti-rabbit fluorescein conjugated antiserum (Cappel), and washed (12 hrs.) in PBS-TA and PBS for (6 hrs. each). The ganglia were then mounted in a 1:9 mixture of 50 mM Tris buffer (pH 9.5):glycerol with 4% n-propyl gallate (Giloh and Sedat, 1982) and viewed with an epifluorescence microscope (Ziess standard with excitation and barrier filters for fluorescein).

For immunostaining of sectioned tissues, ganglia were dissected, fixed, and washed as described above except they were not incubated in protease. Ganglia were then incubated in 20% sucrose PBS until they sank (at least 1 hr.), frozen in O.C.T. Compound (Miles, Inc.), and

cryostat sectioned at 20 μm. The sections were incubated in 20 mM PBS-TA (10 min.), in 6% NGS PBS-TA (20 min.), and in the primary antiserum (1:10) at 4°C (12 hrs.). They were then washed (10 min.) in PBS-TA (2X) before incubating (1 hr.) at room temperature with the secondary antiserum (1:100). The sections were washed (10 min.) in PBS (2X), mounted, and viewed as described above. Specificity of antisera staining was tested by incubating tissues with blocking solution alone or the primary antiserum preabsorbed for 24 hrs. with 100 μM αBCP 1-9 (Pulst et al., 1988). This incubation, however, appeared to diminish but did not completely block staining indicating that the peptide this antiserum recognized in *Archidoris* is not identical to αBCP 1-9 in *Aplysia*.

(ii) Vascular Injection. The extent and pattern of vascularization in the circumesophageal ganglia was visualized by injection of a 4:1 mixture of 5% gelatin (Baker Chem.) and ink (Speedball, water soluble, blue) containing 80 μl of 4% paraformaldehyde per ml. The gelatin and ink mixture was prepared at 37°C, cooled to room temperature, and vortexed with fixative immediately prior to injection through a cannula in the heart. After coagulation the ganglion was dissected and post-fixed in 4% paraformaldehyde PBS.

Electrophysiology: Intra- and extra-cellular recordings of IWCs were obtained from semi-intact preparations of *Archidoris*. For these preparations an animals was cooled to 5°C and anesthetized as described

above before the dorsal body wall, anterior foot, and buccal mass were dissected away so the central ganglia could be viewed by trans- or epi-illumination. The dissected animal was then immobilized in a Sylgard-coated recording chamber and perfused with aerated ASW (room temperature), enriched with 0.2% glucose, Eagles Minimum Medium Essential (0.2X) and Non-Essential (0.2X) amino acid and vitamin solutions (0.5X; Gibco). To expose the IWCs the outer connective tissue sheath was dissected from the ganglia. Glass microelectrodes (10-40 MΩ) filled with 2 M potassium acetate and a glass suction electrode (A-M System, Inc.) filled with ASW were used to intra- and extracellularly monitor IWC activity, respectively.

The IWCs were stimulated both intra- and extracellularly. For intracellular stimulation experiments the inner sheath was dissected away in order to penetrate the IWCs, however, this dissection often damaged the superficial cells in the cluster. To visualize the morphology of intact cells Lucifer Yellow (3% solution in 0.1% LiCl) was iontophoretically injected (hyperpolarizing current pulses of 5-10 nA, 1 s duration, 0.5 Hz) into IWC penetrated after the inner ganglionic sheath was softened by protease crystals (Type IV, Sigma) applied to the sheath for approximately 2 min. A 40 μm coated stainless steel wire was used to extracellularly stimulate the intracerebral commissure or IWC clusters through stimulus

isolation and constant current units (Grass Instruments). All experimental data were recorded on chart (Gould 2400s) and FM tape recorders (Hewlett Packard 3969A).

Egg-laying bioassay: Egg-laying bioactivity of IWC clusters was assayed using the following protocol. Source ganglia for homogenates were removed from animals which had not laid eggs within the previous 48 hrs and immediately placed in ice cold ASW. The cerebral ganglia or IWC clusters and intercerebral commissure were then dissected along with an equal volume of neural tissue taken from the right pedal and buccal ganglia. The latter ganglia lack αBCP immunoreactive neurons or processes and thus serve as a control injection of neural tissues. The cerebral ganglia, IWC clusters, or control ganglia from 2 animals were combined in 100 μl of ASW in a siliconized microcentrifuge tube and homogenized with a plastic pestle. IWC and control homogenates were then injected into the hemocoel of animals through their foot using a 100 μl Hamilton syringe. Animals injected were sexually mature individuals (ranging from 5-20 g) matched for size and weight, which had not laid eggs within the previous 48 hrs. After injection the animals were checked every hour for the presence or absence of egg masses in their container. All egg masses laid within four hours of injection were scored as an egg-laying event.

RESULTS

Morphology

The intercerebral white cells of *Archidoris* are located on the medial surface of the bilateral cerebral ganglia immediately posterior to the cerebral commissure. They form two clusters of about 100 neurons each and appear distinctly white under epi-illumination following removal of the outer connective tissue sheath which surrounds the circumesophageal ganglia (Fig. 4.1, A). Each cluster is a disk of cell bodies one to two cells thick contained within a separate connective tissue sheath. The IWC somata vary approximately two-fold in size, with the larger cells (20 μm in diameter) located medially within the cluster. The circumesophageal ganglia are extensively vascularized, and injection of gelatinous ink into the arterial system shows that the IWC clusters and the intercerebral commissure are located adjacent to a large hemolymph sinus that receives hemolymph directly from the main artery supplying the ganglia (Fig. 4.1, B).

Iontophoretic injection of Lucifer Yellow into individual IWC somata revealed two morphological types of cells (Fig. 4.2). One cell type was unipolar possessing a single process that extended through the commissure to the contralateral IWC cluster. The second type was a bipolar, or bifurcating monopolar, cell with one process that branched

anteriorly into the ipsilateral cerebral ganglion and a second process that extended through the commissure and branched anteriorly into the contralateral cerebral ganglion.

αBCP immunoreactivity

The somata and processes of IWCs are immunoreactive (-ir) for $\alpha BCP_{1\text{-}9}$ (Fig. 4.3 and 4.4), one of the most conserved peptide transmitters synthesized in the ovulogenic neurons of *Aplysia*. In whole ganglia the anterior branching of IWC processes were clearly visible as horn-like projections similar to the morphology exhibited by individual Lucifer Yellow injected neurons (Fig. 4.3, A). In sections the immunoreactive processes from each cluster of neurons extended into the intercerebral commissure and the cerebral ganglia. Alpha BCP-ir processes were most densely distributed in the ventrally in the intercerebral commissure where they collectively form a loop (Fig. 4.3, C). This structure may be a neurohemal release site since dye fixed in the vasculature of sectioned ganglia surrounds the commissure (Fig. 4.3, B). Ventral to the clusters, labeled processes radiate from the commissure, through the cerebral ganglion, and into the ipsilateral pleural ganglion (Fig. 4.3, D).

Alpha BCP-ir neurons were also found outside of the IWC clusters. Several of these "ectopic" neurons were observed just outside of the cluster above the intercerebral commissure (not shown). Two larger (30

μm) αBCP-ir cells were located in the cerebral ganglia anterior to the commissure (Fig. 4.3, E), and one or two others (40 μm) were also present in each pleural ganglion (Fig. 4.3, F). The latter neurons branched extensively throughout the pleural ganglia and into the cerebral ganglia.

Electrophysiological properties

All electrical recordings from IWCs showed them to be silent neurons with large resting membrane potentials. The average potential of the 10 cells with the lowest resting potentials was -66.7 $\pm$ 3.1 mV. The IWCs were refractory to electrical stimulation and spike activity could not be evoked by intracellular (1-10 nA, 50 msec, 1-5 Hz through glass microelectrodes) or extracellular stimulation of the intercerebral commissure or contralateral cluster (50 μA-5 mA, .1-5 msec, 1-5 Hz through a fine stainless steel electrode). Application of pronase crystals also failed to evoke bursts as seen in *Aplysia* or *Lymnaea*. The same protocols for electrical stimulation elicited afterdischarges in BC clusters of *Aplysia* in two preparations used as positive controls for our methods.

Bioactivity

We injected homogenized whole cerebral ganglia or isolated IWC clusters into resting animals to determine whether the IWCs contained transmitters capable of stimulating egg-laying. Homogenates of the cerebral ganglia or IWC clusters (including the intercerebral commissure) from two animals stimulated egg-laying within 2-4 hrs after injection into

quiescent animals. The control homogenates of right pleural and buccal ganglia did not stimulate this behavior (Table 4.1). *Archidoris* normally lay a counterclockwise spiral egg-mass. The mass is composed of capsules containing 1-18 eggs arranged in a string that folds back and forth to form a gelatinous ribbon 1-2 cm in width (McGowan, 1951). The ribbon is attached to the substrate along one side to form a spiralling egg mass. Most of the animals injected with IWC homogenates laid spiral egg-masses identical to this normal pattern. A few injected animals laid short ribbons indicating some deficiency of mature eggs or activity of the injected homogenate. These animals had been held in captivity longer and may not have had adequate nutrient resources to produce the same number of eggs. In any case, the appearance of eggs was scored as a positive result.

DISCUSSION

Ovulogenic neurons thus far described in gastropod molluscs share common properties that may characterize homologous neurons in other molluscan systems. Our results show that the Intercerebral White Cells of the nudibranch *Archidoris montereyensis* share morphological, behavioral, biochemical, and electrophysiological characteristics with the ovulogenic neurons of other gastropods (Table 4.2). Chief among these are their

cellular anatomy, immunocytochemical staining for a conserved peptide expressed by ovulogenic neurons, and their potency in stimulating egg-laying activity.

The IWCs are located in two clusters on the medial surface of each cerebral ganglion. The clusters, like the CDC clusters in *Lymnaea* appear to be composed of cells exhibiting different morphologies which may reflect differences in their function. For example, the cell type with extensive branching into each cerebral ganglion may be the primary mediator of input to and electrical coupling between clusters, while the cell type with one process into the commissure may form the primary peptide release route. The presence of IWC processes with varicosities in the intercerebral commissure of *Archidoris*, and the proximity of this tissue to ganglionic vascular spaces, suggests that the commissure in this animal, like that of *Lymnaea*, may serve as a neurohemal release site.

Homogenates of the IWC and their processes in the intercerebral commissure stimulate egg-laying when injected into quiescent animals as do BC and CDC extracts in *Aplysia* and *Lymnaea*, respectively (Kupfermann, 1967; Toevs and Brackenbury, 1969; Geraerts and Bohlken, 1976; Arch, 1976; Ebberink et al., 1985). Similarities between the induced and natural behaviors in *Archidoris* are unknown, but the form of the induced egg-masses were identical in most instances to those laid

spontaneously. Thus, the egg-laying bioactivity of IWC extracts indicates that hormones functionally similar to the egg-laying hormones in *Aplysia* and *Lymnaea* may be produced by these cells.

The immunoreactivity of the IWCs also indicates that they synthesize egg-laying hormones structurally homologous to those of the BCs and CDCs. Antisera to αBCP, one of the more conserved peptides encoded by the ELH gene of *Aplysia*, labels the IWCs and their processes. The staining was not completely blocked by αBCP preabsorption, however, suggesting that the structure of this peptide in *Archidoris* is not identical.

Alpha-BCP immunoreactivity in central neurons other than the IWCs suggest that the IWCs are components of larger neuroendocrine system controlling egg-laying similar to that of *Aplysia* and *Lymnaea* (Chiu and Strumwasser, 1984; Mahon et al., 1985; Pulst et al., 1988; Van Minnen et al., 1988, 1989). The αBCP immunoreactive neurons in the pleural ganglia are particularly intriguing as they may be homologous to the ELH/αBCP neurons in the pleural ganglia of *Aplysia* that trigger the BC clusters to discharge (Brown et al., 1989). The morphology of the αBCP-ir neurons in *Archidoris* does support this hypothesis as these neurons possess processes that extend into the region of the cerebral ganglia where the IWC processes extensively branch.

Based on homologies discussed thus far, we hypothesized that the

IWCs exhibit a pattern of activity similar to the BCs and CDCs. Normally the electrically coupled BC and CDC neurons are silent with large resting potentials. When spontaneously active prior to egg-laying, or when stimulated with extracellular or intracellular current, BC or CDC afterdischarges last up to 30 and 60 minutes, respectively, after which they are highly refractory to further stimulation (Kupfermann and Kandel, 1970; Vlieger et al., 1980). The IWCs of *Archidoris* show some of these characteristics; they are silent cells with large resting potential, but we were unable to stimulate any form of spike activity in these cells.

The IWCs appear to be another example of ovulogenic neurons controlling egg-laying behavior providing further evidence that this system is evolutionarily conserved within the gastropod molluscs. From this standpoint it is noteworthy that the ganglionic location of the IWCs in *Archidoris* is identical to that of the CDCs of the pulmonate *Lymnaea* rather than that of the BCs in the related opisthobranch *Aplysia*. The location of CDC clusters in the cerebral ganglion appears to be a general property of this system in all pulmonates (Roubos and Van De Ven, 1987; Khan et al., 1990; Van Minnen et al., 1992). Conversely, BCs or putative ovulogenic neurons in other anapsid opisthobranchs like *Aplysia* are generally located in abdominal (fused parietal, intestinal, and visceral ganglia) or visceral ganglia (Pinsker and Dudek, 1977; Dudek et al., 1980; Ram, 1982b). The exception are putative ovulogenic neurons in

Pleurobranchia located in the pedal ganglion (Ram et al., 1977). Ram et al. (1977) postulate the parietal ganglion may have fused with the pedal in this species. This seems unlikely, however, given that in other species the parietal is thought to fuse with the pleural (Bullock, 1965) or cerebral (Page, 1992) ganglia. Although ovulogenic neurons have not been identified in the third gastropod subclass, the prosobranchia, candidate neurons exist in the intestinal ganglia based on their egg-laying bioactivity (Ram, 1977). Thus, it appears that ovulogenic neurons are located in the cerebral ganglia in gastropods that have convergently evolved more consolidated nervous systems, such as the pulmonates and the nudibranch opisthobranchs. Alternatively, in the more primitive anapsid opisthobranchs and prosobranchs that possess less cephalized ganglia, the ovulogenic neurons are located in ganglia of the visceral loop. The position of these cells in visceral loop ganglia appears to be correlated with increased distance between visceral and head ganglia and may reflect the predominant actions of ovulogenic neurons of viscero-motor circuits.

Table 4.1 Induction of egg-laying by cerebral ganglia and IWC cluster homogenates. This table shows the number of animals that laid eggs or failed to lay eggs when injected with the indicated homogenate.

Homogenate	Eggs	No Eggs	Animals (#)
Cerebral ganglia	5*	1	6
Control ganglia	1	5	6
ICW cell clusters	3**	0	3
Control ganglia	0	3	3

* $p < .01$; one-tailed Fisher exact probability test.
** $p < .05$; one-tailed Fisher exact probability test.

Table 4.2 Comparison of morphological, physiolgical, and biochemical characteristics of the BCs of *Aplysia*, the CDCs of *Lymnaea*, and the ICWCs of *Archidoris*.

CHARACTERISTICS	BCs	CDCs	ICWCs
Morphology:			
ganglionic location	abdominal	cerebral	cerebral
vascular spaces	adjacent	adjacent	adjacent
cells/cluster	~400	40-100	25-50
cell size	≤75 μm	≤90 μm	≤20 μm
cell morphology	multipolar	unipolar	unipolar
Bioactivity:			
homogenates	+ egg-laying	+ egg-laying	+ egg-laying
in situ activity	precedes egg-laying	precedes egg-laying	?
Transmitter Phenotype:			
ovulation hormone gene	ELH gene	CDCH gene	?
homologous peptides	1) ELH 2) αBCP 3) ßBCP γBCP 4) δBCP	CDCH αCDCP $ß_{1-3}$CDCP Calfluxin	αBCP-ir
Electrophysiology:			
resting activity	silent	silent	silent
membrane potential	-58--68 mV*	-40--50 mV**	-55--70 mV
afterdischarge	65 ± 21 min	21 ± 13 min	?
electrically coupled	yes	yes	?

+, stimulate

* Kits, 1980; ** Kupfermann and Kandel, 1970

Figure 4.1 Dorsal view of the circumesophageal ganglia showing location of Intercerebral White Cells (IWCs). A. Under epi-illumination the IWCs were visible as two clusters of white cells (arrows) located on the medial surface of the cerebral ganglia (C) immediately below the cerebral commissure (v). Pl, pleural ganglion. (50X) B. The artery supplying the circumesophageal ganglia (arrow) branched ventrally (circle) into the vascular space immediately surrounding the IWC clusters and intercerebral commissure. (32X)

Figure 4.1

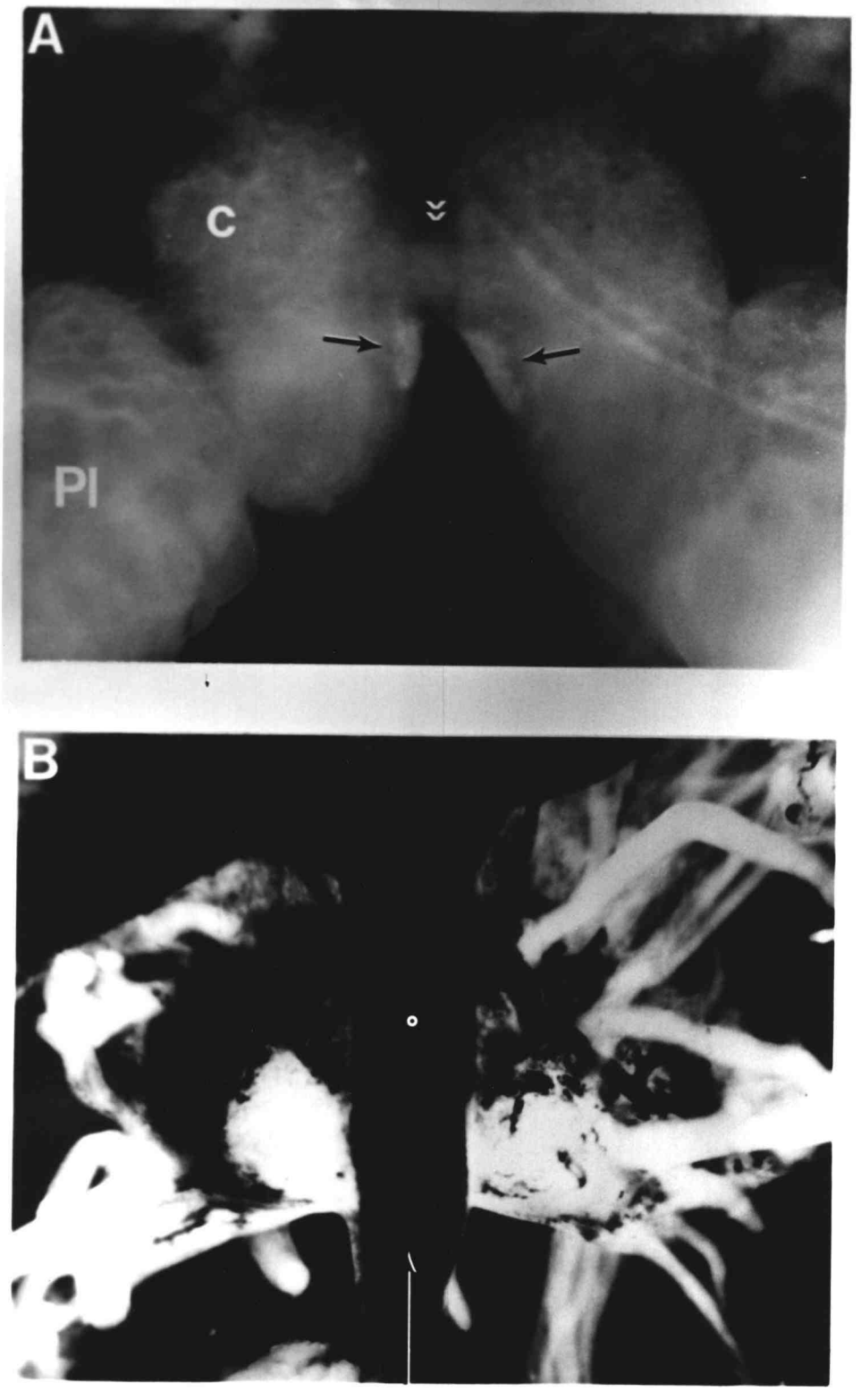


Figure 4.2 Morphology of IWCs. Camera lucida tracing of Lucifer Yellow injected IWCs showing the two IWC morphologies observed. One cell type possessed a single process which extended through the cerebral commissure and terminated in the contralateral IWC cluster. The other cell type had a bifurcating process with one branch extending anteriorly in the ipsilateral cerebral ganglion and the other branch extending through the commissure to the contralateral IWC cluster. This process appeared to extend anteriorly in the cerebral ganglion and terminate in many fine branches, however, the process could not be traced in its entirety and that is represented by a break in the drawing. C, cerebral ganglion; comm, intercerebral commissure. Scale = 40 μm.

Figure 4.2

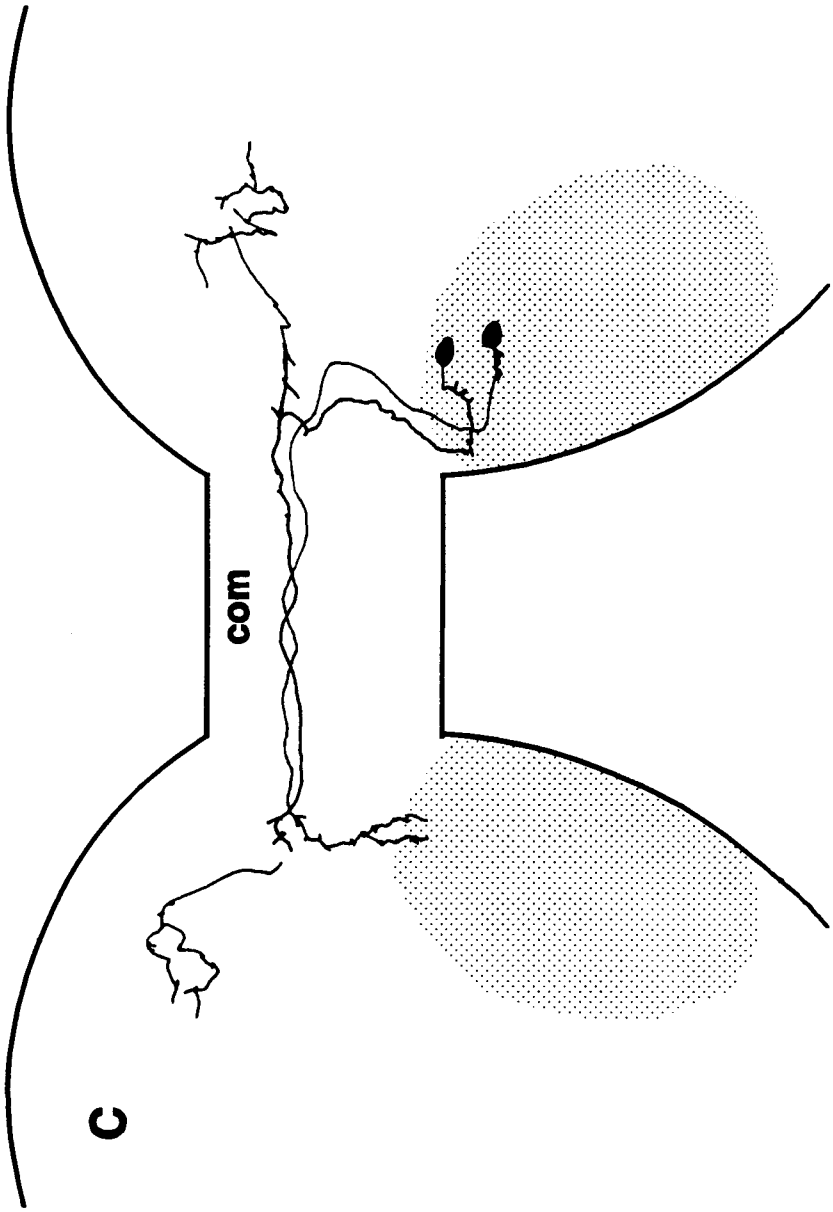


Figure 4.3 The IWCs and their processes were immunoreactive for αBCP. A. Photomicrograph of the entire circumesophageal ganglion showing the position of αBCP-ir cells and processes in the cerebral ganglion. (Scale = 200μm) B-F. Photomicrographs of sectioned ganglion showing αBCP-ir neurons. The IWCs and their processes were αBCP-ir and surrounded by vascular spaces (arrows; B). Collectively, the labeled processes formed a looped structure (arrow) in the intercerebral commissure (C). Ventrally these processes extended through the cerebral ganglion and into the pleural ganglion (D). Each cerebral ganglion contained a larger immunoreactive cell, like this one in the right hemiganglion, anterior and lateral to the commissure (E). Additionally, each of the pleural ganglia contained one or two αBCP-ir cells. These cells were larger than the IWCs and their processes branched extensively throughout the pleural ganglia (F). C, cerebral ganglia; Pl, pleural ganglia. Scale = 25 μm.

Figure 4.3

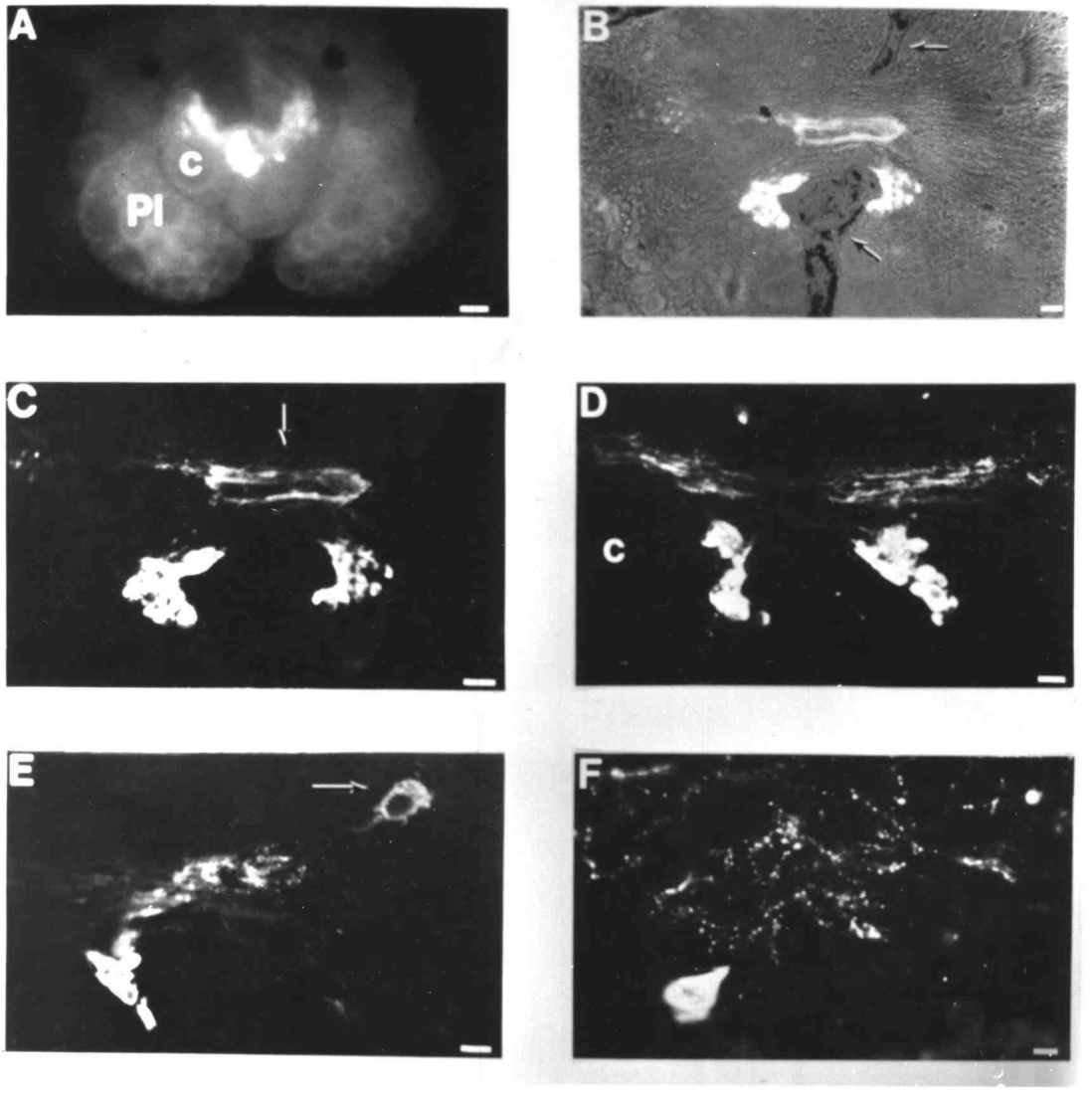
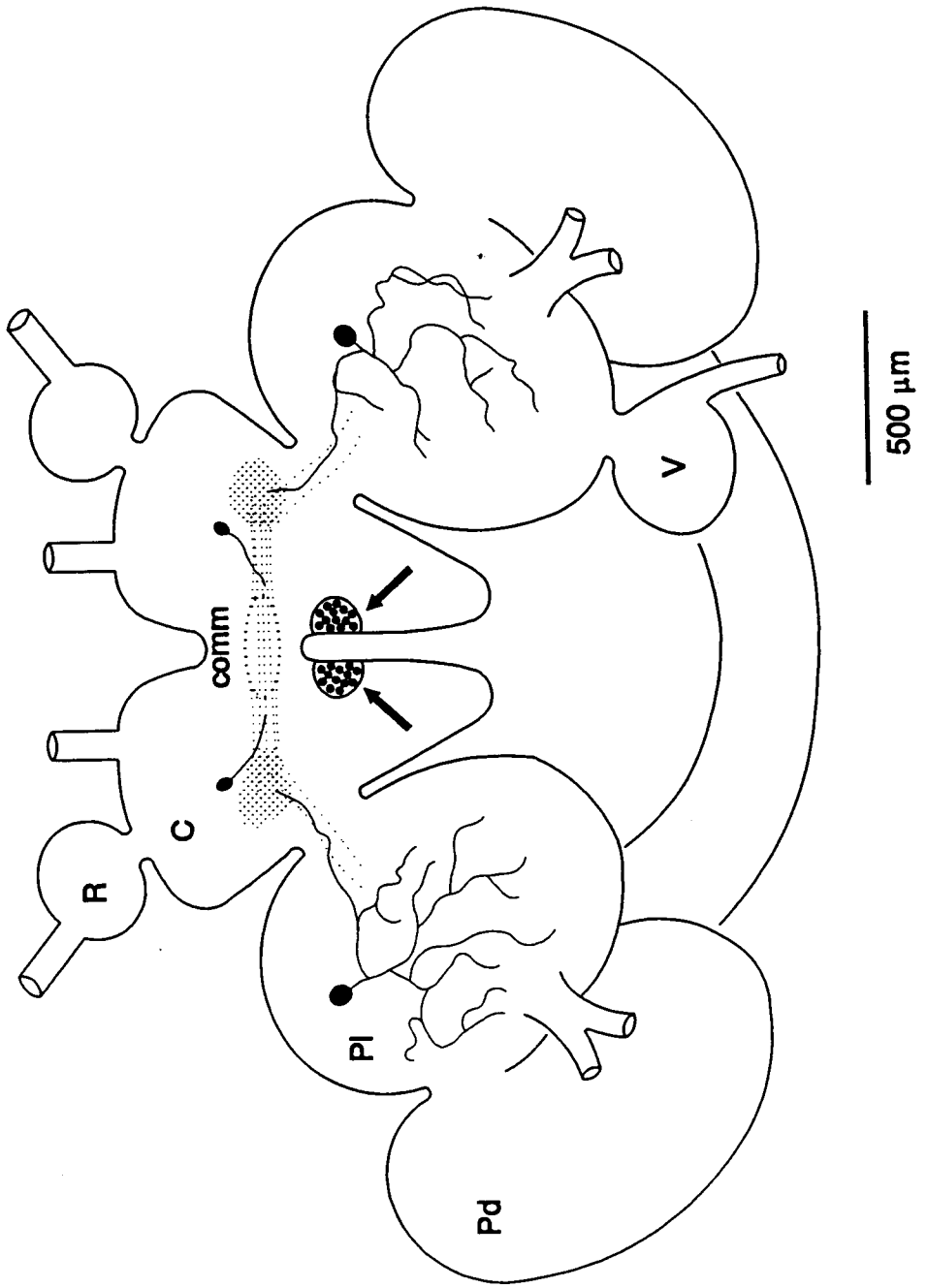


Figure 4.4 Schematic diagram of αBCP-ir neurons and processes in the circumesophageal ganglia. Alpha BCP-ir neurons included the IWC clusters (arrows) immediately posterior to the intercerebral commissure (comm) in the cerebral ganglia (C), a larger neuron ($\sim$30 μm) in each cerebral ganglion with a process that extended into the commissure, and 1-2 larger neurons in each pleural ganglion (Pl). The pleural neurons branched throughout the pleural ganglion and into the cerebral ganglion where they met branching IWC processes (stippled area). Pd, pedal ganglion; R, rhinophore ganglion; V, visceral ganglion.

Figure 4.4



CHAPTER 5

DISCUSSION

The research in this thesis describes a visceral muscle preparation - the heart of the nudibranch *Archidoris montereyensis* - that is an especially suitable model system in which to answer questions concerning the behavioral relevance of transmitter interactions. In this discussion I will review the characteristics of this system that make it an amenable model.

1) The motor circuit regulating the heart is uniquely simple and effective. As described in the introduction to this thesis, the heart of *Archidoris*, like that of other gastropods, is innervated by a small group of excitatory and inhibitory motor neurons (Wiens and Brownell, 1990a). Two of the five motor neurons innervating the heart - the excitor, PI_{HE}, and the inhibitor, PI_{HI} in the right pleural ganglion- are major effectors of cardiac activity and the most potent cardiac motor neurons described in molluscs. Three additional motor neurons in the visceral ganglion - the excitor, V_{HE}, and the inhibitors, $VHI_{1\ and\ 2}$ - have weaker actions on the heart, comparable to the actions of heart motor neurons in other gastropods. Morphological evidence indicates that these visceral motor neurons may receive synaptic

input from the pleural heart motor neurons as the axons of the latter branch extensively in the visceral ganglion. The actions of the pleural heart motor neurons and the extent and complexity of their axonal and dendritic processes suggest that neural pathways affecting cardiac function will impinge upon these cells. Thus, it should be possible to characterize neuronal processes that regulate cardiac responses during specific behaviors by monitoring and manipulating the activity in these identified pleural heart motor neurons.

2) Multiple transmitter systems converge onto the heart. The heart is responsive to both amine and peptide transmitters. As in other gastropods, the biogenic amines, 5HT and DA, and ACh have excitatory and inhibitory actions, respectively. The peptides, R15α2, SCP_B, myomodulin, and FMRFa all increase the rate and amplitude of heart contractions. These transmitters appear to be endogenous cardio-regulators due to their presence in the nervous and cardiovascular systems, however, none of them are detectable in the identified cardiac motor neurons in the pleural ganglion. Two conclusions regarding the complexity of cardiac regulation can be made from these results. First, additional, perhaps novel, cardio-regulatory transmitters must mediate the actions of the pleural heart motor neurons. Secondly, other neurons must innervate the heart as the identified motor neurons are not the source of

the transmitter immunostaining observed in the heart. These neurons, most likely neurosecretory, may affect other parameters of cardiac function besides amplitude and rate. Thus, as in other gastropods (Koester and Koch, 1987), several classes of neurons, including excitatory and inhibitory motor and neurosecretory neurons, may innervate the heart.

Interestingly, despite the number of active transmitters described, all of the excitatory transmitters have similar chronotropic and inotropic actions, with the latter being the most predominant. Assuming that each of these transmitters is an endogenous cardio-regulatory agent, the purpose of employing so many transmitters with similar functions is not clear. My data suggests that while their *in vitro* actions are similar, their function *in vivo* may be more specific. First, the different activity thresholds exhibited by each transmitter, combined with unique anatomical differences in release may provide for specificity of action in some cases. For example, it is reasonable to predict that the high thresholds of myomodulin and FMRFa, and the regional distribution of processes immunoreactive for these transmitters, could contribute to limiting the actions of these transmitters to specific regions of the heart. Secondly, specificity of action may result through interactions between transmitters. For example, I showed that myomodulin which has a high threshold of activity when applied alone to the heart, acts at a lower concentration to potentiate the inotropic component of the heart's

response to 5HT. Myomodulin is unique, thus far, in terms of its ability to modulate the actions of 5HT on the heart of *Archidoris*. Perhaps more can be learned about interactions between transmitters by monitoring parameters of cardiac function that precede changes in overt cardiac function, such as second messenger activation or alteration in ionic conductances. Subtle effects of modulatory transmitters on cardiac function would be missed using our current isolated heart assay because activity of the heart was often inconsistent, making small changes in response difficult to quantify.

Studies of gastropod somatic muscles show that pre- and post-synaptic interactions between transmitters are a common mode of transmitter action (Weiss et al., 1992). The physiological significance of theses interactions remains unclear, however, because behaviors using these muscles cannot be evoked in experimental preparations. Weiss and his co-workers (1992) recently hypothesized that peptide transmitters released simultaneously with conventional transmitters act to fine-tune the motor output of the muscles. Thus, interactions between transmitters may provide a finer degree of control than the simple on/off switch provided by the motor neurons. The cardiac neuromuscular preparation of *Archidoris* appears to be an advantageous system to test this hypothesis. The heart and the neurons innervating it are robust and maintain activity for many hours in dissected, "behaving" preparations of the animal.

3) Egg-laying behavior in *Archidoris* is induced by a discrete group of neuroendocrine cells that may act on the cardiovascular system.

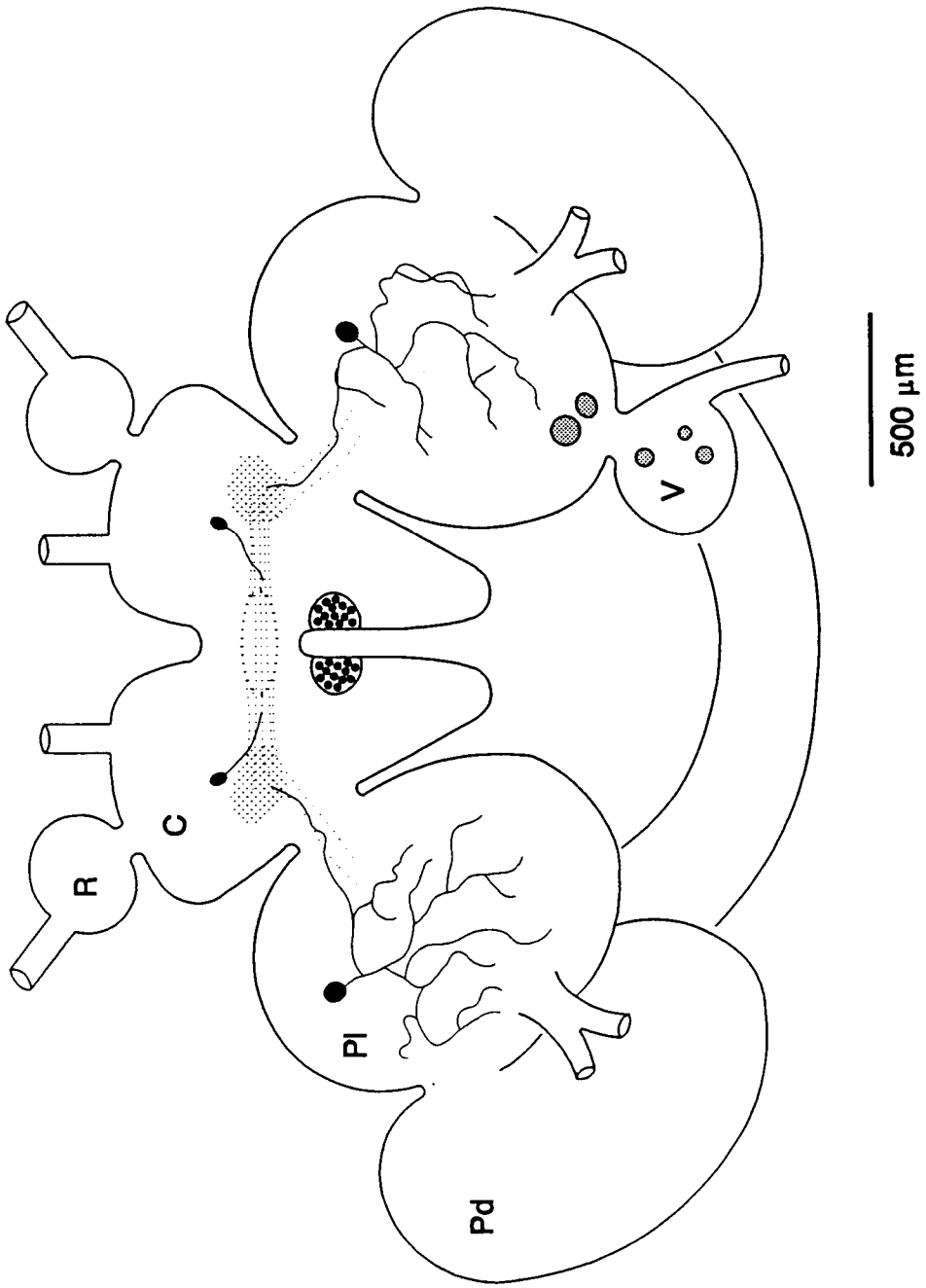
Investigation of the neural mechanism of cardiac regulation in the context of egg-laying is a promising experimental approach because the neural correlates of this behavior can be studied in dissected preparations of the animal. The IWCs in *Archidoris* share morphological, biochemical, and electrophysiological properties with ovulogenic neuroendocrine systems in the opisthobranch, *Aplysia*, and the pulmonate, *Lymnaea* suggesting that the same neuroendocrine mechanisms for initiating egg-laying behavior exist in all gastropods. Several cardiovascular changes are hypothesized to occur during egg-laying in *Aplysia* based on the effects of bag cell peptides on cardiovascular neurons. The role of individual neurons and transmitters in this response is not understood, however, due to the difficulty encountered in maintaining experimental preparations of the *Aplysia* heart and weak coupling between motor neuron and cardiac activity. Using *Archidoris*, though, we will be able to ask questions concerning behavioral relevance of cardiac motor neuron activity and the actions of cardio-regulatory transmitters.

The pattern of αBCP immunostaining in the central nervous system of *Archidoris* suggests that an interaction exists between the IWCs and heart motor neurons. The larger αBCP immunoreactive neurons in the pleural ganglia branched extensively throughout the pleural ganglia.

These processes in the right pleural ganglion extended into the region of the pleural heart motor neurons where they may synaptically or non-synaptically influence the activity of these neurons (Fig. 5.1). Therefore, once the stimulation parameters that elicit IWC activity are determined, *Archidoris* should be a particularly useful model to study neural regulation of the heart in the context of a complex, natural behavior.

Figure 5.1 Schematic diagram showing the positions of α-BCP immunoreactive neurons (solid) and heart motor neurons (stippled) in the central nervous system of *Archidoris*. C, cerebral ganglion; Pd, pedal ganglion; Pl, pleural ganglion; R, rhinophore ganglion; V, visceral ganglion.

Figure 5.1



LITERATURE CITED

Abrams, T.W., Castellucci V.F., Camardo J.S., Kandel E.R., Lloyd P.E. 1984. Two endogenous neuropeptides modulate the gill and siphon withdrawl reflex in *Aplysia* by presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel. Proc. Natl. Acad. Sci. 81:7956-7960.

Alevizos A., Bailey C.H., Chen M., Koester J. 1989. Innervation of vasculature and cardiac muscle of *Aplysia* by multimodal motoneuron L7. J Neurophysiol. 61:1053-1063.

Alevizos A., Karagogeos D., Weiss K.R., Buck L., Koester J. 1991a. R15a1 and R15α2 peptides from *Aplysia*: Comparison of bioactivity, distribution, and function of two peptides generated by alternative splicing. J. Neurobiol. 22(4)405-417.

Alevizos A., Weiss K.R., Koester J. 1991b. Synaptic actions of identified peptidergic neuron R15 in *Aplysia*. I. Activation of respiratory pumping. J. Neurosci. 11:1263-1274.

Alevizos A., Weiss K.R., Koester J. 1991c. Synaptic actions of identified peptidergic neuron R15 in *Aplysia*. II. Contraction of pleuroabdominal connectives mediated by motoneuron L7. J. Neurosci. 11:1275-1281.

Alevizos A., Weiss K.R., Koester J. 1991d. Synaptic actions of identified peptidergic neuron R15 in *Aplysia*. III. Activation of the large hermaphroditic duct. J. Neurosci. 11:1282-1290.

Arch S. 1976. Neuroendocrine regulation of egg laying in *Aplysia californica*. Amer. Zool. 16:167-175.

Arch S., Smock T. 1977. Egg-laying behavior in *Aplysia californica*. Behav. Biol. 19:45-54.

Arshavsky Y.I., Deliagina T.G., Gelfand I.M., Orlovsky G.N., Panchin Y.V., Pavlova G.A., Popova L.B. 1990. Neural control of heart beat in the pteropod mollusc *Clione limacina*: Coordination of circulatory and locomotor systems. J. Exp. Biol. 148:461-475.

Bloom F.E. 1988. Neurotransmitters: past, present, and future directions. FASEB 2:32-41.

Bogerd J., Geraerts W.P.M., Van Heerikhuizen H., Kerkhoven R.M., Joosse J. 1991. Characterization and evolutionary aspects of a transcript encoding a neuropeptide precursor of *Lymnaea* neurons, VD1 and RPD2. Brain Res. 11:47-54.

Boyd, P.J., Gardner C.R., Walker R.J. 1985. Actions of some 5-hydroxytryptamine analogues on the isolated heart of the snail *Helix aspersa*. Comp. Biochem. Physiol. 81:233-239.

Branton D.W., Arch S., Smock T., Mayeri E. 1978. Evidence for mediation of a neuronal interaction by a behaviorally active peptide. Proc. Natl. Acad. Sci. 75:5732-5736.

Brown R.O., Mayeri E. 1989. Positive feedback by autoexcitatory neuropeptides in neuroendocrine bag cells of *Aplysia*. J. Neurosci. 9:1443-1451.

Brown R.O., Pulst S.M., Mayeri E. 1989. Neuroendocrine bag cells of *Aplysia* are activated by bag cell peptide-containing neurons in the pleural ganglion. J. Neurophysiol. 61(6):1142-1152.

Brownell P.H. 1983. Neuroendocrine mechanisms of visceromotor behavior in *Aplysia*. *In: Molluscan Neuroendocrinology.* J. Lever and H.H. Boer (eds.) North-Hollan Pub. Co., Amsterdam. pp. 256-263.

Brownell P.H., Ligman S.H. 1992. Mechanisms of circulatory homeostasis and response in Aplysia. Experientia. In Press.

Brownell P.H., Morgan J.L.M., Ligman S.H. 1992. A survey of peptide actions on motoneurons regulating circulatory and respiratory functions in *Aplysia*. *In: Phylogenetic Models in Functional Coupling of the CNS and the Cardiovascular System.* R.B. Hill, K. Kuwasawa, B.R. McMahon, T. Kuramoto (eds.) Comp. Physiol. Basel, Karger. vol. 11, pp. 199-210.

Buck, L.B., Bigelow J.M., Axel R. 1987. Alternative splicing in individual *Aplysia* neurons generates neuropeptide diversity. Cell. 51:127-133.

Buckett K.J., Dockray G.J., Osborne N.N., Benjamin P.R. 1990a. Pharmacology of the myogenic heart of the pond snail *Lymnaea stagnalis*. J. Neurophysiol. 63:1413-1425.

Buckett K.J., Peters M., Dockray G.J., Van Minnen J., Benjamin P.R. 1990b. Regulation of heartbeat in *Lymnaea* by motoneurons containing FMRFamide-like peptides. J. Neurophysiol. 63:1427-1435.

Buckett K.J., Peters M., Benjamin P.R. 1990c. Excitation and inhibition of the heart of the snail, *Lymnaea*, by non-FMRFamidergic motoneurons. J. Neurophysiol. 63:1436-1447.

Bulloch T.H. 1965. Mollusca: Gastropoda. *In: Structure and Function in the Nervous System of Invertebrates (II).* T.H. Bullock, G.A. Horridge (eds.) W.H. Freeman and Company, San Francisco and London. Chap. 23, pp. 1283-1386.

Bulloch A.G.M., Price D.A., Murphy A.D., Lee T.D., Bowes H.N. 1988. FMRFamide peptides in *Helisoma*: Identification and physiological actions at a peripheral synapse. J. Neurosci. 8:3459-3469.

Burnstock G. 1987. Present status of purinergic neurotransmission-implications for vascular control. *In: Neuronal Messengers in Vascular Function.* A. Nobin, C. Owman, B. Arneklo-Nobin (eds.) Elsevier, Amsterdam. Chap. 22, pp. 327-340.

Byrne J.H. 1983. Identification and initial characterization of a cluster of command and pattern-generating neurons underlying respiratory pumping in *Aplysia californica*. J Neurophysiol. 49(2):491-508.

Byrne J.H., Koester J. 1978. Respiratory pumping: neuronal control of a centrally commanded behavior in *Aplysia*. Brain Res 143:87-105.

Calabrese R.L. 1989. Modulation of muscle and neuromuscular junctions in invertebrates. Sem. Neurosci. 1:25-34.

Campanelli J.T., Scheller R.H. 1987. Histidine-rich basic peptide: A cardioactive neuropeptide from *Aplysia* neurons R3-14. J. Neurophysiol. 57(4):1201-1209.

Chiu A.Y., Strumwasser F. 1984. Two neuronal populations in the head ganglia of *Aplysia californica* with egg-laying hormone-like immunoreactivity. 294:83-93.

Coggeshall R.E. 1972. The muscle cells of the follicle of the ovotestis in *Aplysia* as the probable target organ for bag cell extract. Am. Zool. 12:521-523.

Coggeshall R.E., Kandel E.R., Kupfermann I., Waziri R. 1966. A morphological and functional study on a cluster of identifiable neurosecretory cells in the abdominal ganglion of *Aplysia californica*. J. Cell. Biol. 31:363-368.

Cottrell G.A., Osborne N. 1969. A neurosecretory system terminating in the *Helix* heart. Comp. Biochem. Physiol. 28:1455-1459.

Dalsgaard C.J., Franco-Cereceda A., Saria A., Lundberg J.M., Theodorsson-Norheim E., Hokfelt T. 1986. Distribution and origin of substance P- and neuropeptide Y- immunoreactive nerves in the guinea-pig heart. Cell Tiss. Res. 243:477-485.

Dieringer N., Koester J., Weiss K.R. 1978. Adaptive changes in heart rate of *Aplysia californica*. J. Comp. Physiol. 123:11-21.

Dockray G.J., Reeve J.R., Shively J., Gayton R.J., Barnard C.S. 1983. A novel active pentapeptide from chicken brain identified by antibodies to FMRFa. Nat. 305:328-330.

Drummond G.I., Wernham S., Lukowiak K. 1985. Stimulation of adenylate cyclase in the heart of *Aplysia californica* by biogenic amines. Comp. Biochem. Physiol. 80(1):129-133.

Dudek F.E., Weir G., Acosta-Urquidi J., Tobe S.S. 1980. A secretion from neuroendocrine bag cells evokes egg release *in vitro* from ovotestis of *Aplysia californica*. Gen. Comp. Endocrinol. 40:241-244.

Ebberink R.H.M., van Loenhout H., Geraerts W.P.M., Joosse J. 1985. Purification and amino acid sequence of the ovulation neurohormone of *Lymnaea stagnalis*. Proc. Nat. Acad. Sci. USA. 82:7767-7771.

Feinstein R., Pinsker H., Schmale M., Gooden B.A. 1977. Bradycardial response in *Aplysia* exposed to air. J. Comp. Physiol. 122:311-324.

Frazier W.T., Kandel E.R., Kupfermann I., Waziri R., Coggeshall E. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. J. Neurophys. 30:1288-1351.

Fujimoto K., Kubota I., Yasuda-Kamatani Y., Minakata H., Nomoto K., Yoshida M., Harada A., Muneoka Y., Kobayashi M. 1991. Purification of achatin-I from the atria of the african giant snail, *Achatina fulica*, and its possible function. Biochem. and Biophys. Res. Comm. 177:948-853.

Furgal S.M., Brownell P.H. 1987. Ganglionic circulation and its effects on neurons controlling cardiovascular functions in *Aplysia californica*. J. Exp. Zool. 244:347-363.

Furukawa Y., Kobayashi M. 1987a. Neural control of heart beat in the african giant snail, *Achatina fulica* ferussac I. Identification of the heart regulatory neurones. J. Exp. Biol. 129:279-293.

Furukawa Y., Kobayashi M. 1987b. Neural control of heart beat in the african giant snail, *Achatina fulica* ferussac II. Interconnections among the heart regulatory neurons. J. Exp. Biol. 129:295-307.

Ganong W.F. 1989. *Review of medical physiology.* 14th ed. Appleton and Lange, Norwalk, Connecticut. Chaps. 31 and 33, pp. 503-513, 533-546.

Geraerts W.P.M., Bohlken S. 1976. The control of ovulation in the hermaphroditic freshwater snail *Lymnaea stagnalis* by the neurohormone of the caudodorsal cells. Gen. Comp. Endocrinol. 28:350-357.

Geraerts W.P.M., Cheeseman P., Ebberink R.H.M., Nuyt K., Hogenes TH.M. 1983. Partial purification and characterization of the ovulation hormone of the freshwater pulmonate snail *Lymnaea stagnalis*. Gen. Comp. Endocrinol. 51:471-476.

Geraerts W.P.M., Hogenes Th.M. 1985. Heterogeneity of peptides released by electrically active neuroendocrine caudodorsal cells of *Lymnaea stagnalis*. Brain Res. 331:51-61.

Geraerts W.P.M., Ter Maat A., Vreugdenhil E. 1988. The peptidergic neuroendocrine control of egg-laying behavior in *Aplysia* and *Lymnaea*. *In: Endocrinology of Selected Invertebrate Types.* H. Laufer, R.G.H. Downer, (eds.) Alan R. Liss, Inc., NY. pp. 141-231.

Giloh H., Sedat J.W. 1982. Fluorscence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Sci. 217:1252-1255.

Grega D.S., Prior D.J. 1985. The effects of feeding on heart activity in the terrestrial slug, *Limax maximus*: central and peripheral control. J. Comp. Physiol. A. 156:539-545.

Guyton A.C. 1981. *Textbook of medical physiology*. Sixth ed. W.B. Saunders Company, Philadelphia, PA. Chap. 13, p. 158.

Hall J.D., Lloyd P.E. 1990. Involvement of pedal peptide in locomotion in *Aplysia*: Modulation of foot muscle contractions. J. of Neurobiol. 21(6):858-868.

Harris L., Ono J.K. 1990. The distribution of immunoreactive FMRFamide in neuronal fibers, varicosities, and somata in the heart of the sea hare, *Aplysia californica.* Neurosci. Abstr. 16:549.

Hill R.B. 1974. Effects of 5-hydroxytryptamine on action potentials and on contractile force in the ventricle of *Dolabella auricularia*. J. Exp. Biol. 61:529-539.

Hokfelt T. 1987. Neuronal communication through multiple coexisting messengers. *In: Synaptic Function.* G.M. Edelman, W.E. Gall, W.M. Cowan (eds.) John Wiley and Sons, New York. Chap. 7, pp. 179-211.

Jones H.D. 1983. The circulatory systems of gastropods and bivalves. *In: The Mollusca (v. 5)*. K.M. Wilbur, A.S.M. Saleuddin (eds.) Academic Press, New York, pp. 189-238.

Josse J. 1988. The hormones of molluscs. *In: Endocrinology of Selected Invertebrate Types.* H. Laufer, R.G.H. Downer, (eds.) Alan R. Liss, Inc., pp. 89-140.

Kaczmarek L.K., Finbow M., Revel J.P., Strumwasser F. 1979. The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. J. Neurobiol. 10:535-550.

Kerkhoven R.M., Croll R.P., Van Minnen J., Bogerd J., Ramkema M.D., Lodder H., Boer H.H. 1991. Axonal mapping of the giant peptidergic neurons VD1 and RPD2 located in the CNS of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain Res. 565:8-16.

Khan H.R., Ashton M.L., Mukai S.T., Saleuddin A.S.M. 1990. The effects of mating on the fine structure of neurosecretory caudodorsal cells in *Helisoma duryi* (Mollusca). Can. J. Zool. 68:1233-1240.

Kits K.S. 1980. States of excitability in ovulation hormone producing neuroendocrine cells of *Lymnaea stagnalis* (Gastropoda) and their relation to the egg-laying cycle. J. Neurobiol. 11(4):397-410.

Koch U.T., Koester J., Weiss K.R. 1984. Neuronal mediation of cardiovascular effects of food arousal in *Aplysia*. J. Neurophysiol. 51(1):126-135.

Kobayashi M. 1987. Innervation and control of the heart of a gastropod, *Rapana*. Experientia 43:981-985.

Kobayashi M., Muneoka Y. 1989. Functions, receptors, and mechanisms of the FMRFamide-related peptides. Biol. Bull. 177:206-209.

Kobayashi M., Muneoka Y. 1990. Structure and action of molluscan neuropeptides. Zool. Sci. 7:801-814.

Koester J. 1989. Chemically and electrically coupled interneurons mediate resperatory pumping in *Aplysia*. J. Neurosci. 9:4078-4088.

Koester J., Alevizos A. 1989. Innervation of the kidney of *Aplysia* by L10, the LUQ cells, and an identified peripheral motoneuron. J. Neurosci. 9:4078-4088.

Koester J., Koch U.T. 1987. Neural control of the circulatory system of *Aplysia*. Experentia 43:972-980.

Koester J., Mayeri E., Liebeswar G., Kandel E.R. 1974. Neural control of circulation in *Aplysia*. II. Interneurons. J. Neurophysiol. 37(3):476-496.

Kreiner T., Rothbard J.B., Schoolnik G.K., Scheller R.H. 1984. Antibodies to synthetic peptides defined by cDNA cloning reveal a network of peptidergic neurons in *Aplysia*. J. Neurosci. 4(10):2581-2589.

Kupfermann I. 1967. Stimulation of egg laying: Possible neuroendocrine function of bag cells of the abdominal ganglion of *Aplysia californica.* Nat. 216:814-815.

Kupfermann I. 1970. Stimulation of egg laying by extracts of neuroendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. J Neurophysiol. 33:877-881.

Kupferman I., Kandel E.R. 1970. Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (Bag Cells) in abdominal ganglion of Aplysia. J. Neurophysiol. 33:865-876.

Kuwasaua, K. 1979. Effects of Ach and IJPs on the Av valve and the ventricle of *Dolabella auriculana*. Amer. Zool. 19:129-143.

Liebeswar G., Goldman J., Koester J., Mayeri E. 1975. Neural control of circulation in *Aplysia*. III. Neurotransmitters. J. Neurophys. 38(4):767-779.

Ligman S.H., Brownell P.H. 1985. Differential hormonal activation of the bag cell neurons on the arterial system of *Aplysia*. J. Comp. Physiol. 157:31-37.

Lloyd P.E. 1982. Cardioactive neuropeptides in gastropods. FASEB 41(13)2948-2952.

Lloyd P.E. 1986. The small cardioactive peptides: A class of modulatory neuropeptides in *Aplysia*. Trends Neurosci. 9:428-431.

Lloyd P.E., Kupfermann I., Weiss K.R. 1984. Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in *Aplysia*. Proc. Natl. Acad. Sci. 81:2934-2937.

Lloyd P.E., Kupfermann I., Weiss K.R. 1985. Two endogenous neuropeptides (SCP_A and SCP_B) produce a cAMP-mediated stimulation of cardiac activity in *Aplysia*. J. Comp. Physiol. 156:659-667.

Longley R.D., Longley A.J. 1986. Serotonin immunoreactivity of neurons in the gastropod *Aplysia californica*. J. Neurobiol. 17:339-358.

McCaman M.W., Ono J.K., McCaman R.E. 1979. Dopamine measurements in molluscan ganglia and neurons using a new, sensitive assay. J. Neurochemistry. 32:1111-1113.

MacKay A.R., Gelperin A. 1972. Pharmacology and reflex responsiveness of the heart in the giant garden slug, *Limax maximus*. Comp. Biochem. Physiol. 43A:877-896.

Mahon A.C., Nambu J.R., Taussig R., Shyamala M., Roach A., Scheller R.H. 1985a. Structure and expression of the egg-laying hormone gene family in *Aplysia*. J. Neurosci. 5:1872-1880.

Mahon, A.C., Lloyd P.E., Weiss K.R., Kupfermann I., Scheller R.H. 1985b. The samll cardioactive peptides A and B of *Aplysia* are derived from a common precursor molecule. Proc. Natl. Acad. Sci. USA 82:3925-3929.

Manabe N., Foldes F.F., Torocsik A., Nagashima H., Goldiner P.L., Vizi E.S. 1991. Presynaptic interaction between vagal and sympathetic innervation in the heart: modulation of acetylcholine and noradrenaline release. J. Autonomic Nerv. Sys. 32:233-242.

Masinovsky, B., Kempf S.C., Callaway J.C., Willows A.O.D. 1988. Monoclonal antibodies to the molluscan small cardioactive peptide SCPB: Immunolabeling of neurons in diverse invertebrates. J. Comp. Neurol. 272:500-512.

Mayeri E., Brownell P., Branton W.D. 1979a. Multiple, prolonged actions of neuroendocrine bag cells on neurons in *Aplysia*. I. Effects on bursting pacemaker neurons. J. Neurophys. 42:1165-1184.

Mayeri E., Brownell P., Branton W.D. 1979b. Multiple, prolonged actions of neuroendocrine bag cells on neurons in *Aplysia*. II. Effects on beating pacemaker and silent neurons. J. Neurophys. 42(4):1185-1197.

Mayeri E., Koester J., Kupfermann I., Liebeswar G., Kandel E.R. 1974. Neural control of circulation in *Aplysia*. I. Motoneurons. J. Neurophysiol. 37(3):458-475.

Mayeri E., Rothman B.S., Brownell P.H., Branton W.D., Padgett L. 1985. Nonsynaptic characteristics of neurotransmission mediated by egg-laying hormone in the abdominal ganglion of *Aplysia*. J. Neurosci. 5(8):2060-2077.

McAdoo D.J., Iliffe T.M., Price C.H., Novak R.A. 1978. Specific glycine uptake by identified neurons of *Aplysia californica*. II. Biochemistry. Brain Res. 154:41-51.

McGowan J.A. 1951. The early embryology of the nudibranch *Archidoris montereyensis* Macfarland. M.S. Thesis, Oregon State University, Corvallis, OR. pp. 50.

Miller, M.W., Alevizos, A., Cropper, E.C., Vilim, F.S., Karagogeos, D., Kupfermann, I., Weiss, K.R. 1991a. Localization of myomodulin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica*. J. Comp. Neurol. 314:627-644.

Miller M.W., Stamm S., Cropper E.C., Vilim F.S., Beushausen S., Brosius J., Kupfermann I., Weiss K.R. 1991b. Characterization of a cDNA clone encoding multiple myomodulin-related neuropeptides in *Aplysia*. Neurosci. Abstr. 17:1305.

Mukai S.T., Saleuddin A.S.M. 1989. Mating increases the synthetic activity on the neurosecretory caudodorsal cells of *Helisoma duryi* (Mollusca:Pulmonata). Can. J. Zool. 67:2363-2367.

Nambu J.R., Scheller R.H. 1986. Egg-laying hormone genes of *Aplysia*: Evolution of the ELH gene family. Neurosci. 6(7):2026-2036.

Nambu J.R., Taussig R., Mahon A.C., Scheller R.H. 1983. Gene isolation with cDNA probes from identified *Aplysia* neurons: Neuropeptide modulators of cardiovascular physiology. Cell 35:47-56.

O'Donohue T.L., Bishop J.F., Chronwall B.M., Groome J., Watson W.H.III. 1984. Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system. Peptides 5:563-568.

Ono J.K., McCaman R.E. 1984. Immunocytochemical localization and direct assays of serotonin-containing neurons in *Aplysia*. Neurosci. 11:549-56

Owman C., Chang J., Ekblad E., Steinbusch H.W.M. 1987. Immunohistochemical investigation of the relationship between different neuropeptides and amine transmitters in monkey and guinea-pig cerebral arteries. *In: Neuronal Messengers in Vascular Function.* A. Nobin, C. Owman, B. Arneklo-Nobin (eds.) Elsevier, Amsterdam. Chap. 24, pp. 353-370.

Page L.R. 1992. New interpretation of a nudibrach central nervous system based on ultrastructural analysis of nuerodevelopment in *Melibe leonina*. I. Cerebral and visceral loop ganglia. Biol. Bull. 182:348-365.

Painter S.D., Chong M.G., Wong M.A., Gray A., Cormier J.G., Nagle G.T. 1991. Relative contributions of the egg layer and egg cordon to pheromonal attraction and the induction of mating and egg-laying behavior in *Aplysia*. Biol. Bull. 181:81-94.

Painter S.D., Gustavson A.R., Kalman V.K., Nagle G.T., Blankenship J.E. 1989. Induction of copulatory behavior in *Aplysia*: Atrial gland factors mimic the excitatory effects of freshly deposited egg cordons. Behavioral and Neural Biol. 51:222-236.

Pappano A.J., Mubagwa K. 1992. Actions of muscarinic agents and adenosine on the heart. *In: The heart and cardiovascular system*, 2nd ed. H.A. Fozzard (ed.) Raven Press, Ltd., New York. Chap. 70, pp. 1765-1776.

Pinsker H.M., Dudek F.E. 1977. Bag cell control of egg laying in freely behaving *Aplysia*. Sci. 197:490-493.

Potter E.K. 1985. Prolonged non-adrenergic inhibition of cardiac vagal action following sympathetic stimulation: neuromodulation by neuropeptide Y? Neurosci. Lett. 54:117-121.

Price C.H., McAdoo D.J. 1979. Anatomy and ultrastructure of the axons and terminals of neurons R3-R14 in *Aplysia*. J. Comp. Neurol. 188:647-677.

Price C.H., Coggeshall R.E., McAdoo D.J. 1978. Specific glycine uptake by identified neurons of *Aplysia californica*. I. Autoradiography. Brain Res. 154:25-40.

Price D.A., Greenberg M.J. 1989. The hunting of the FaRPs: The distribution of FMRFamide-related peptides. Biol. Bull. 177:198-205.

Pulst S.-M., Gusman D., Mayeri E. 1988. Immunostaining for peptides of the egg-laying hormone/bag cell peptide precursor protein in the head ganglia of *Aplysia*. Neurosci. 27:363-371.

Ram J.L. 1977. Hormonal control of reproduction in *Busycon*: laying of egg capsules caused by nervous system extracts. Biol. Bull. 152:221-232.

Ram J.L. 1982a. Hormonal control of reproduction in *Busycon*: II. Laying of egg-containing capsules caused by nervous system extracts and further characterization of the substance causing egg capsule laying. Biol. Bull. 162:360-370.

Ram J.L. 1982b. Interspecific induction of egg laying in Hawaiian *Aplysiids*. Gen. Comp. Endo. 47:522-528.

Ram J.L., Salpeter S.R., Davis W.J. 1977. Pleurobranchaea egg-laying hormone: localization and partial purification. J. Comp. Physiol. 119:171-194.

Rittenhouse A.R., Price C.H. 1985. Peripheral axons of the parabolic burster neuron R15. Brain Res. 333:330-335.

Rittenhouse A.R., Price C.H. 1986. Electrophysiological and anatomical identification of the peripheral axons and target tissues of *Aplysia* neurons R3-14 and their status as multifunctional, multimessenger neurons. J. Neurosci. 6(7):2071-2084.

Roubos E.W., Van De Ven A.M.H. 1987. Morphology of neurosecretory cells in basommatophoran snails homologous with egg-laying and growth hormone-producing cells of *Lymnaea stagnalis*. Gen. Comp. Endocrinol. 67:7-23.

Rothman B.S., Mayeri E., Brown R.O., Yuan P., Shively J.E. 1983a. Primary structure and neuronal effects of α-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia*. Proc. Natl. Acad. Sci. 80:5753-5757.

Rothman B.S., Weir G., Dudek F.E. 1983b. Egg-laying hormone: Direct action on the ovotesetis of *Aplysia*. Gen. Comp. Endocrinol. 52:134-141.

S.-Rozsa K. 1979a. Heart Regulatory neural network in the central nervous system of *Achatina fulica* (ferussac). Comp. Biochem. Physiol. 63A:435-445.

S.-Rozsa K. 1979b. Analysis of the neural network regulating the cardio-renal system in the central nervous system of *Helix pomatia* L. Amer. Zool. 19:117-128.

S.-Rozsa K., Salanki J., Vero M., Kovacevic N., Konjevic D. 1980. Neural network regulating heart activity in *Aplysia depilans* and its comparison with other gastropod species. Comp. Biochem. Physiol. 65A:61-68.

Said S.I. 1987. Neuropeptides in cardiovascular regulat*ion. In: Brain Peptides and Catecholamines in Cardiovascular Regulation.* J.P. Buckley and C.M. Ferrario, (eds.) Raven Press, New York.

Sawada M., McAdoo D.J., Ichinose M., Price C.H. 1984. Influences of glycine and neuron R14 on contraction of the anterior aorta of *Aplysia*. Jap. J. Physiol. 34:747-767.

Schaefer M., Picciotto M.R., Kreiner T., Kaldany R.R., Taussig R. Scheller R.H. 1985. *Aplysia* neurons express a gene encoding multiple FMRFamide neuropeptides. Cell 41:457-467.

Scheller R.H., Jackson J.F., McAllister L.B. Rothman B.S., Mayeri E., Axel R. 1983. A single gene encodes multiple neuropeptides mediating a stereotyped behavior. Cell 32:7-22.

Skelton M.E., Koester J. 1991. Innervation of the heart of *Aplysia* by serotonin and R15α2 peptide-immunoreactive terminals, and by neuron L2. Neurosci. Abstr. 17:1355.

Skelton M.E., Weiss K.R., Koester J. 1990. The serotonergic heart excitor neuron, RB_{HE} of *Aplysia californica* synthesizes R15α2 peptide. Neurosci. Abstr. 16:549.

Sloley B.D., Juorio A.V., Durden D.A. 1990. High-performance liquid chromatographic analysis of monoamines and some of their gamma-glutamyl conjugates produced by the brain and other tissues of *Helix aspersa* (Gastropoda). Cell and Mol. Neurobiol. 10(2):175-192.

Smith P.J.S. 1985. Cardiac performance in response to loading pressures in *Busycon canliculatum* (Gastropoda) and *Mercenaria mercenaria* (Bivalvia). J. Exp. Biol. 119:301-320.

Smith P.J.S. 1990. Integrated cardiovascular control in the mollusca. Physiological Zoology 63:12-34.

Smith P.J.S., Hill R.B. 1987. Modulation of output from an isolated gastropod heart: Effects of acetylcholine and FMRFamide. J. Exp. Biol. 127:105-120.

Smock T., Arch S., Lloyd P. 1978. Secretory sources of egg-laying induction also influence the isolated *Aplysia* heart. Soc. Neurosci. Abstr. 4:206.

Soinila S., Mpitsos G.J. 1991. Immunohistochemistry of diverging and converging neurotransmitter systems in mollusks. Biol. Bull. 181:484-499.

Storm-Mathisen J., Ottersen O.P. 1986. Antibidies against amino acid neurotransmitters. *In: Neurohistochemistry: Modern Methods and Applications.* P. Panula, H. Paivarinta, S. Soinila (eds.) Alan R. Liss, New York. pp. 107-136.

Stuart D.K., Chiu A.Y., Strumwasser F. 1980. Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of *Aplysia*. J. Neurophysiol. 43:488-498.

Sundler F., Edvinsson L., Ekblad E., Hakanson R., Uddman R. 1987. Existence and coexistence of peptides in perivascular nerve fibres. *In: Neuronal messengers in vascular function*. A. Nobin, C. Owman, B. Arneklo-Nobin (eds.) Elsevier, Amsterdam. Chap. 23, pp. 341-353.

Takeda N. 1977. Stimulation of egg-laying by nerve extracts in slugs. Nat. 267:513-514.

Ter Maat A., Dijcks F.A., Bos N.P.A. 1986. In vivo recordings of neuroendocrine cells (caudo-dorsal cells) in the pond snail. J. Comp. Physiol. A 158:853-859.

Ter Maat A., Geraerts W.P., Jansen R.F., Bos N.P. 1988. Chemically mediated positive feedback genrates long-lasting afterdischarge in a molluscan neuroendocrine system. Brain Res. 438:77-82.

Toevs L.A., Brackenbury R.W. 1969. Bag cell-specific proteins and the humoral control of egg-laying in *Aplysia californica*. Comp. Biochem. Physiol. 29:207-216.

Umitsu Y., Matsumoto H., Koike H. 1987. Active contraction of nerve bundle and identification of a nerve-contractor motorneuron in *Aplysia*. J. Neurophysiol. 58:1016-1034.

Van Minnen J., Dirks R.W., Vreugdenhil E., Van Diepen J. 1989. Experession of the egg-laying hormone genes in peripheral neurons and exocrine cells in the reproductive tract of the mollusc *Lymnaea stagnalis*. Neurosci. 33:35-46.

Van Minnen J., Haar Ch.v.d., Rapp A.K., Vreugdenhil E. 1988. Localization of ovulation hormone-like neuropeptide in the central nervous system of the snail *Lymnaea stagnalis* by means of immunocytochemistry and in situ hybridization. Cell Tissue Res. 251:477-484.

Van Minnen J., Schallig H.D.F.H., Ramkema M.D. 1992. Identification of putative egg-laying hormone containing neuronal systems in gastropod molluscs. Gen. and Comp. Endocrin. 86:96-102.

Vanhoutte P.M., Levy M.N. 1980. Prejunctional cholinergic modulation of adrenergic neurotransmission in the cardiovascular system. Am. J. Physiol. 238:H275-H281.

Vatner S.F. 1992. Sympathetic mechanisms regulating myocardial contractility in conscious animals. *In: The Heart and Cardiovascular System*, 2nd ed. H.A. Fozzard et al. (eds.) Raven Press, Ltd., New York. Chap. 67, pp. 1709-1728.

Vlieger T.A., Kits K.S., Ter Maat A., Lodder J.C. 1980. Morphology and electrophysiology of the ovulation hormone producing neuro-endocrine cells of the freshwater snail *Lymnaea stagnalis* (L.). J. Exp. Biol. 84:259-271.

Vreugdenhil E., Jackson J.F., Bouwmeester T., Smit A.B., Van Minnen J.V., Heerikhuizen H.V., Klootwijk J., Joosse J. 1988. Isolation, characterization, and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the freshwater snail *Lymnaea stagnalis*. J. Neurosci. 8:4184-4191.

Walker R.J. 1986. Transmitters and modulators. *In: The Mollusca*. A.O. Willows (ed.) Academic Press, New York. Vol. 9, pp. 279-485.

Warner M.R., Levy M.N. 1988. Neuropeptide Y as a putative modulator of the vagal effects on heart rate. Circ. Res. 64:882-889.

Weinreich D., McCaman M.W., McCaman R.E., Vaughn J.E. 1973. Chemical, enzymatic and ultrastructural characterization of 5-hydroxytryptamine-containing neurons from the ganglia of *Aplysia californica* and *Tritonia diomedia*. Neurochem. 20:969-976.

Weiss K.R., Brezina V., Cropper E.C., Hooper S.L., Miller M.W., Probst W.C., Vilim F.S., Kupfermann I. 1992. Peptidergic co-transmission in *Aplysia*: Functional implications for rhythmic behaviors. Experientia 48:456-463.

Weiss K.R., Cohen J.L., Kupfermann I. 1978. Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. J. Neurophysiol. 41(1):181-203.

Wernham S., Lukowiak K. 1983. The effect of serotonin, dopamine and arginine vasotocin on the isolated *Aplysia* heart-I. Heart rate and amplitude of contraction. Comp. Biochem. Physiol. 75:295-303.

Welsford I.G., Prior D.J. 1991. Modulation of heart activity in the terrestrial slug *Limax maximus* by the feeding motor program, small cardioactive peptides and stimulation of buccal neuron B1. J. Exp. Biol. 155:1-19.

Wiens B.L., Brownell P.H. 1989. Cardiac innervation in the nudibranch mollusc, *Archidoris montereyensis*. Neurosci. Abst. 15(2):1298.

Wiens B.L., Brownell P.H. 1990a. Characterization of cardiac innervation in the nudibranch, *Archidoris montereyensis*. J. Comp. Physiol. 167:51-60.

Wiens B.L., Brownell P.H. 1990b. The distribution of cardioactive transmitters in the heart and nervous system of the nudibranch, *Archidoris montereyensis*. Neurosci. Abstr. 16(1):549.

Wiens B.L., Brownell P.H. 1991. The actions of mollscan cardioactive peptides on the heart of the nudibranch, *Archidoris montereyensis*. Neurosci. Abstr. 17(2):1356.

Williams R.G., Dockray G.J. 1983. Immunohistochemical studies of FMRF-amide-like immunoreactivity in rat brain. Brain Res. 276:213-229.

Yatani A., Okabe K., Codina J., Birnbaumer L., Brown A.M. 1990. Heart rate regulation by G proteins acting on the cardiac pacemaker channel. Sci. 249:1163-1168.